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L17	L5 same (cancer$1 or tumor$1 or tumour$1 or malignan$4 or neoplastic or carcinoma$1 or adenocarcinoma$1)	1490	L17
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L15	l5 same ((immune or immunotherap$3 or vaccine$1 or vaccination or lymphocyte$1 or neutrophil$2 or macrophage$1 or monocyt$2 or ((T or B or NK) adj cell$1)))	1318	L15
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Photodynamic therapy and Immune response in tumor bearing mice

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ABSTRACT

Since immune response of the host is important in the control of tumor growth and spreading, and the Photodynamic therapy (PDT) is able to increase the antitumor immunity, in our laboratory we examine the effect of PDT on immune compartment of tumor bearing mice. Lymphocytes and macrophages collected from tumor bearing mice pretreated with PDT are cytotoxic in vitro and in vivo against the parental tumor lines,in contrast the same immune cells population collected from tumor bearing mice pretreated only with laser light are unable to lyse the parental tumor cells. In adoptive immunotherapy experiments, treatment of mice bearing MS-2 tumor with adoptive transfer of immune lymphocytes collected from mice pretreated with PDT is able to significantly increase the survival time;in contrast the lymphocytes collected from mice pretreated only with laser light were not able to modify the survival time suggesting that the laser treatment alone did not increase the immune response of the host. In conclusion these results demonstrate that the PDT induce a strong immune response on the host and the stimulated lymphocytes generated could be use for an adoptive immunotherapy approach; moreover laser treatment alone (thermal effect) is unable to modulate the immune response of the host.

Keywords: Photodynamic therapy, Phthalocianines, Laser light, Lymphocytes, Macrophages,Adoptive transfer

1. INTRODUCTION

A major goal of cancer treatment is selective destruction of malignant cells with preservation of normal tissues and functions. Photodynamic therapy (PDT) destroys malignant tumors through preferential uptake by neoplastic cells of photosensitizing compounds, which are then activated by suitable light exposure[1,2]. Activated photosensitizers interact with molecular oxygen to produce singlet oxygen that destroys neoplastic cells with minimal normal tissue damage [3]. In vitro and in vivo studies indicate that PDT kill cells both directly and indirectly as a result of reduced blood flow in and towards the tumor [4].

PDT has been demonstrated to be effective in eradicating transplanted tumors in experimental animals[1]. PDT utilizing the hematoporphyrin derivative (Hpd) has been used clinically for palliation of obstructive lesions of the esophagus[5] and the tracheobronchial tree[6], for treatment of bladder tumors[7] and for local control of various tumors on the skin surface[8]. Persistent cutaneous photosensitivity,known as a major side effect of systemic PDT,may lead to erythema,or blistering of light-exposed skin,if patients ignore the proper protection from sunlight[9]. This acute inflammatory reaction and the histological changes of PDT-treated tisssue with infiltrated lymphocytes,plasma cells and histiocytes[10],suggest a mayor immunological component in the response after photosensitization. There are indications that the tumoricidal activity of these activated

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inflammatory cells makes an essential contribution to the antitumor effect of PDT[11]. Increased macrophage activity was demonstrated after PDT in vitro and in vivo[12]. It was also reported that macrophages release tumor necrosis factor alpha (TNF-α) following PDT treatment[13] and the same PDT treatment altered the expression of citokines Interleukin 6 and Interleukin 10[14]. We have recently shown that PDT is able to induce a strong antitumor immunity in tumor bearing mice[15] and that the lymphocytes population can play an important role in the PDT modulation of immune response[16].

In contrast, in preliminary experiments, we observe that laser light treatment alone is not able to induce a host immune reaction. Aim of this research is to study the effect of PDT with Aluminum Phthalocyanines (AlS_2Pc) and laser light on lymphocytes and macrophages in vivo and to utilize these activated immune lymphocytes for adoptive immunotherapy experiments,furthermore to analyze if laser light treatment without the sensitizer is able to increase the functional activity of lymphocytes and macrophages.

2. MATERIAL AND METHODS

2.1 Animals and Tumor

Inbred Balb/c and hybrid DBA/2 x Balb/c male mice,8-10 weeks old,obtained from Charles River,Calco,Italy were used and hereafter called Balb and CDF1 respectively.

MS-2 fibrosarcoma,originally induced by the Moloney murine sarcoma virus was maintained in the laboratory by weekly i.m. passage of tumor cell homogenate into the right hind leg of Balb mice.[17]

L1210 murine leukemia was obtained from the Italian tumor Institute(Milano,Italy) and maintained by intraperitoneal (i.p.) injection of 10^6 cells/mouse in CDF1 male mice.

For the experiments,tumor from mice were removed under sterile conditions. The cells suspension was obtained by Potter homogenization,counted under optical light microscopy and injected intradermally (i.d. 10^6 cells/mouse). Treatment started when the tumor mass measured approximately 1 cm in diameter. The animals were injected i.v. with the drug and irradiated with the laser 24 hrs later or irradiated only with the same power dose. A single light irradiation was done in all experiments.

2.2 Chemicals

Aluminum phthalocyanine with an average degree of sulphonation of 2.1 (hereafter called AlS_2Pc), was kindly provided by Dr. A. McLennan (Paisley College of Technology, Paisley,U.K.). It was dissolved in physiological solution at a concentration of 5 mg/cc.

2.3 Laser source

Irradiation was applied with a continuous wave dye (DCM) laser (Coherent Mod. CR-599,Palo Alto,CA) pumped by an Argon laser (Coherent Mod. Cr-18,Palo Alto, CA) and tuned at 670 nm. The laser output was coupled to a 400 μm plastic-glass optical fiber (Quartz at Silice PCS600,Paris,France). The laser power was monitored at the fiber output.

2.4 Cytolytic assay

The lytic activity of lymphocytes was evaluated by the ^{51}Cr-release microassay[18]. Briefly,10^4 ^{51}Cr-labelled MS-2 cells in 100μl were mixed with 100μl of effector cells at different concentrations in microtiter plates and incubated 4 hr. at 37° in a moist atmosphere of 95% air and 5%CO2,other samples were incubated with detergent and then the plates were centrifuge and 100μl of the supernatant were counted in an automatic γ-counter. The percentage of specific lysis was calculated as:

$$\%\text{lysis} = \frac{\text{cpm in experimental samples} - \text{cpm in controls}}{\text{cmp in detergent samples} - \text{cpm in control}} \times 100$$

2.5 Tumoricidal assay of activated macrophages

Peritoneal cells from CDF1 mice treated with PDT,with laser light,with AlS_2Pc or untreated were harvested on the 4 th day. The cells were plated on coverglasses in wells and incubated for 1 hour. The non adherent cells were removed and the adherent cells,the macrophages, after intensive washing, were cocultered for 2 hours with target tumor cells. After this incubation the mixtures,macrophages – tumor cells, were injected intradermally,at different concentrations, in syngeneic mice.

2.6 Winn Assay

The in vivo antitumor activity of T cells was determined by the Winn tumor neutralization assay.[19] Effector lymphocytes,collected from mice untreated or treated with PDT,with laser light alone and with AlS_2Pc alone were mixed with MS-2 tumor cells in 0.1 ml PBS and then inoculated intradermally in syngeneic mice.

2.7 Adoptive immunotherapy

Spleen cells from PDT tumor treated tumor bearing mice,or laser light treated tumor bearing mice or virgin mice were collected,washed and $20x10^6$ cell/mouse were inoculated i.v. in recipient mice as reported previously[20].

3. RESULTS AND DISCUSSION

The cytolytic activity of lymphocytes obtained from spleeens of MS-2 fibrosarcoma bearing mice pretreated with PDT or with AlS_2Pc alone or with laser light alone were tested, in vitro,in a 51Cr release assay. The PDT treatment is able to generate cytotoxic lymphocytes against MS-2 target cells instead the treatment with only the sensitizer or the laser light is ineffective (Table 1)

Table 1
Cytotoxicity of immune lymphocytes

Target cells	Effector cells % lisi	
	80 : 1	40 : 1
MS - 2	[a]30.8	25.4
MS - 2	[b]2.4	1.2
MS - 2	[c]1.9	2.1

a = Lymphocytes collected from mice bearing MS-2 and pretreated with PDT (AlS_2Pc + laser light).
b = Lymphocytes collected from mice bearing MS-2 and pretreated with AlS_2Pc.
c = Lymphocytes collected from mice bearing MS-2 and pretreated with laser light.

After this cytotoxic experiment in vitro we tested the activity of macrophages and lymphocytes in vivo to study the influence of PDT or laser light treatment on host immune response. Macrophages collected from PDT pretreated mice were cytotoxic against MS-2 tumor cells (Table 2); in fact the mixture of activated macrophages and tumor cells (10^4) were unable to grow in 2/5 syngeneic mice. For 10^5 tumor cells we observed a remarkable increase of survival time. In contrast macrophages collected from AlS_2Pc alone or laser light alone pretreated mice were completely ineffective in vivo against the MS-2 tumor cells.

Table 2
In vivo cytotoxicity of activated macrophages

Groups	MS-2 cells/mouse		MA cells/mouse	MST	D/T
a_1	10^4	+	0	59	5/5
b_2	10^4	+	10^7	65	5/5
c_3	10^4	+	10^7	61	5/5
d_4	10^4	+	10^7	-	2/5
a_5	10^5	+	0	49	5/5
b_6	10^5	+	10^7	56	5/5
c_7	10^5	+	10^7	52	5/5
d_8	10^5	+	10^7	80	5/5

a= MS-2 cells injected i.d. in CDF1 mice.
b= MS-2 cells mixed with macrophages (MA) collected from mice bearing MS-2, pretreated with AlS_2Pc and injected i.d. in CDF1 mice.
c= MS-2 cells mixed with macrophages (MA) collected from mice bearing MS-2, pretreated with laser light, and injected i.d. in CDF1 mice.
d= MS-2 cells mixed with macrophages (MA) collected from mice bearing MS-2, pretreated with PDT,and injected i.d. in CDF1 mice.

After the demonstration of the cytotoxic activity in vivo of PDT macrophages we wanted to study if also the PDT lymphocytes are immune against the parental tumor line. In a Winn Assay experiment we observed that only spleen lymphocytes from PDT pretreated animals were cytotoxic against the parental line while lymphocytes collected from mice pretreated with the sensitizers alone or with the laser light alone were not cytotoxic and all the animals died with tumor (Table 3)

Table 3
Immune lymphocytes cytotoxicity in vivo (winn assay) MS-2

L	Tumor cells	N° of mice with tumors
L_{PDT}	MS-2	0/5
L_{AlS_2Pc}	MS-2	5/5
L_{laser}	MS-2	5/5

Lymphocytes ($20x10^5$) mixed with tumor cells (10^5) were inoculated i.d. on day 0
L_{PDT}= spleen lymphocytes collected from MS-2 bearing mice pretreated with PDT.
L_{AlS_2Pc}= spleen lymphocytes collected from MS-2 bearing mice pretreated with AlS_2Pc.
L_{laser}= spleen lymphocytes collected from MS-2 bearing mice pretreated with laser light.

The same results we obtaine with another tumor line the L1210 leukemia. Table 4 show that only PDT pretreated lymphocytes were cytotoxic in vivo against the leukemic cells;in contrast the other lymphocytes populations from laser light pretreated animals were ineffective.

Table 4
Immune lymphocytes cytotoxicity in vivo (winn assay)
L1210

IL	N° of mice with tumors
N	5/5
I_L	5/5
I_{PDT}	3/5

Immune lymphocytes ($20x10^5$) mixed with L1210 cells (10^5) were inoculated i.d. on day 0.
N = normal spleen lymphocytes collected from virgin mice.
I_L = spleen lymphocytes collected from L1210 bearing mice pretreated with laser light.
I_{PDT} = spleen lymphocytes collected from L1210 bearing mice pretreated with PDT.

After these encouraging results we want to utilize these immune lymphocytes for an adoptive transfer therapeutical approach. Animals inoculated with 10^3 MS-2 fibrosarcoma tumor cells and treated with lymphocytes collected from PDT pretreated MS-2 bearing mice were unable to survive indefinitely. In contrast the adoptive immunotherapy treatment with lymphocytes collected fronm virgin mice or mice pretreated with only laser light were unable to cure the animals (Table 5).

Table5
Adoptive immunotherapy with immune lymphocytes

N° of tumor cells	Treatment							
MS-2	Untreated		N		I_L		I_{PDT}	
	MST	D/T	MST	D/T	MST	D/T	MST	D/T
10^3	62	5/5	63	5/5	59	5/5		0/5
10^4	56	5/5	54	5/5	53	5/5	-	2/5
10^5	45	5/5	47	5/5	50	5/5	89	3/5

CDF_1 mice challenged i.d. with MS-2 fibrosarcoma on day 0.
N = normal spleen lymphocytes, collected from virgin mice, injected i.v. on day + 1 ($2x10^7$cells).
I_L = spleen lymphocytes collected from MS-2 bearing mice treated with laser light,injected iv. on day + 1 ($2x\ 10^7$cells).
I_{PDT}= spleen lymphocytes, collected from MS-2 bearing mice pretreated with PDT,injected i.v. on day + 1 ($2x10^7$ cells).

Photodynamic therapy,which can effectively destroy malignant tissue,also induce a complex immune response that potentiates the antitumor immunity. Different immune populations are involved in this phenomenon and our results demonstrated that both the macrophages and the spleen lymphocytes from PDT pretreated animals are strongly cytotoxic in vitro and in vivo. Moreover these PDT immune lymphocytes could be use for an adoptive transfer therapeutical approach. In

conclusion the results obtained in the present study confirm the ability of PDT to induce a strong host immune response and suggest the possibility to combine the Photodynamic therapy with an adotive immunotherapy with immune lymphocytes.
In contrast laser light treatment alone (Thermal effect) is completely ineffective for this experimental tumor.

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Contents

DOSE-RELATED IMMUNOLOGICAL AND MORPHOLOGICAL CHANGES OBSERVED IN RATS WITH WALKER-256 CARCINOSARCOMA AFTER PHOTODYNAMIC THERAPY: A CONTROLLED STUDY

F. V. Dima*, V. Vasiliu†, I. N. Mihailescu†, S. V. Dima*, M. Stirbet†, A. Popa† and D. Lacky‡

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‡Victor Babes Institute, Department of Histopathology

Experiments were performed on six batches of Wistar inbred rats with Walker-256 carcinosarcoma 7 days post-transplantation. Animals from batches I and II were exposed to photofrin II (20 mg/kg body mass) or HeNe laser (10 mW; 632.8 nm), respectively; the animals in the batches III–V were given photofrin II, intraperitoneally, 24 h before 60-min laser treatment; one, three and six photofrin/laser treatments, respectively, were applied at an interval of 3 days. The control batch (batch VI) consisted of animals presenting with untreated Walker-256 tumours. The results were as follows: photofrin II or HeNe laser alone (photoexposure to low doses of 15 J/cm²) had no significant effects on tumoural volume and the survival of the rats. Photoexposure to multiple doses of PDT led to complete regression of tumoural volume (65.8%); the cure rate was 31.5% and concomitantly survival rates increased. Cell-mediated immunity tests (performed at 7 and 28 days post-treatment) underlined superior values in batch IV and V animals photoexposed to multiple PDT doses, in comparison with immuno-suppression noticed in batches I–III and the control batch VI. Data presented in this work demonstrate that photodynamic treatment exposure using multiple doses stimulates cell-mediated antitumoural activity, induces modifications in tumoural histological structure, increases survival rates and reduces tumoural incidence in Walker-256 carcinosarcoma in the rat model.

KEY WORDS Photodynamic therapy Walker-256 carcinosarcoma Photofrin II HeNe laser Immune response Concanavalin A

Introduction

Destruction of malignant tumours, previously sensitized with haematoporphyrin derivative, by photodynamic treatment, represents a comparatively new method of tumour therapy, especially in Romania. Haematoporphyrin derivative and photofrin II purified product are complex mixtures of dissolved monomeric porphyrins.[1,2] Both preparations, haematoporphyrin and photofrin II, have antitumoural activities[3] when activated by visible light. Although haematoporphyrin derivative (HpD) was the first to be used in clinics, photofrin II is more active, both against murine tumours[4] and human ones.[3] The high efficacy of photofrin II is determined by the release of biologically inactivated monomeric porphyrins by normal tissues and also by their localization in high amounts within tumoural tissues.[1]

Photodynamic therapy (PDT) of malignant tumours includes biological, photochemical and photophysical processes.[5] These processes involve; (a) absorption of photosensitizing agent; (b) selective retention of the photosensitizer in tumours; (c) irradiation of sensitized tumour by laser radiation.[6] Although many factors determining the response to PDT have been investigated, there are still areas in PDT biology that need further study. Thus, photoimmunologic effects of photodynamic therapy have been studied to some degree, but in other studies cell-mediated anti-tumoural immunity has been systematically investigated after local X-ray irradiation.[7–10] In the present work we study histopa-

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thological modifications which appeared in Walker-256 carcinosarcoma and immunological reactivity of animals after local exposure to photodynamic therapy.

Materials and Methods

Animals, Tumour and Phototherapy

Animals and Tumour Implantation. Walker-256 carcinosarcoma was received from Oncology Institute, Bucharest, Romania and maintained by serial transplantation in inbred Wistar rats. Large tumours were dissected under sterile conditions, macroscopically viable tumour tissue was minced with scissors and forced through sterile needles of decreasing dimensions. Tumoural suspension (0.2 ml) was subcutaneously injected in the dorsal region of syngeneic animals. Tumours were visible in 7 days after transplant and had a volume between 80–120 mm^3. At the start of the study, the animals were 8–10 weeks old, weighing 150–180 g. The animals were from Cantacuzino Institute farms and were kept in subdiet and water *ad libitum*.

Photosensitizer. Photofrin II (Photofrin Medical Inc. Cheetkowage, N.Y.). Rats were intraperitoneally injected with a drug dose of 20 mg/kg body mass. Twenty-four hours post-injection, rats were photoirradiated.

Laser Phototherapy. Our light source was a divergent beam from an LG HeNe Laser (632.8 nm; 10 mW) whose output was checked periodically with a Spectra Physics C power meter.

Experimental Design. Five different modes of treatment were evaluated: Photofrin II only (batch I); HeNe laser only (batch II); PDT with an energy density of 15 J/cm^2 (batch III); PDT at a dose of 30 J/cm^2 (3 exposures × 60 min each: batch IV); PDT at a dose of 60 J/cm^2 (6 × 60 min each, batch V) and the control animals, saline injection, no laser irradiation (batch VI). Fractionated irradiation for animals in batches IV and V was performed at 3 days interval. Each batch included 25 animals. The rats were regularly assessed and tumour size was measured (in mm) along three orthogonal diameters (D1; +D2; +D3), every third day, in the first 2 weeks and then weekly till treatment end (60 days). The volume (V) was calculated assuming spheroid geometry using the following formula:

$$V = \left(\frac{\Pi}{6}\right) D1 \times D2 \times D3 \text{ mm}^3$$

Immunological Tests

Mitogenic Response of Spleen Cells. Splenocytes from all six batches and also from a seventh control batch consisting of 27 healthy animals (not inoculated with saline or treated with HPD) were fractionated on a Nylon column according to the method of Julius *et al.*,[11] at 7 and 28 days post-treatment. The resultant enriched T-cells (Nylon wool-nonadherent) were cultured at a density of 2.5×10^5 cells/well, in RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM glutamine, 50 μ/ml penicillin G, 50 μg/ml streptomycin and 10% foetal calf serum (Gibco, U.K., Europe). With or without the addition of Con A (Concanavalin A, Pharmacia Fine Chemical AB, Uppsala, Sweden), 5 μg/ml in triplicate wells, the cultures were incubated at 37°C in a CO_2 incubator (5% CO_2 + 95% air) for 48 h; 20 μl aliquots of [^{3}H]-thymidine (spec. act. 20 Ci/mmole IFA, Bucharest, Romania) (5 μCi/ml), in RPMI medium were added to the well. After 48-h cultivation, the cells were harvested on glass filter papers, and washed with 5% trichloroacetic acid, methanol dried and then introduced into vials containing 10 ml of scintillation liquid using 0.4% PPO (2,5-diphenyl-oxazole) and 0.01% POPOP (1,4,bis-[5-phenyloxazolyl]-benzene) solution in toluene. The radioactivity was read in a Beckman spectrometer.

Cytotoxicity Assay. Effector cells cytotoxicity was assayed by [^{3}H]-uridine method described previously by Nishimura *et al.*[12] at 7 and 28 days post-treatment. Briefly, aliquots (0.1 ml) of effector cells suspension were mixed with 0.1 ml [^{3}H]-uridine (10 μCi/ml) (spec. act. 30 Ci/mmole, IFA, Bucharest, Romania) labelled target cells (2.5×10^5/0.1 ml) on Coster 96-well round bottom plates at 200:1 effector to target ratio and cultured for 18 h at 37°C. The cells were harvested with the harvester and then the retained radioactivity determined by standard scintillation technique. Target cells lysis was calculated by the following equation:

$$\% \text{ cytotoxicity} = \frac{1\text{-[cpm in culture of effector and target cells]}}{\text{cpm culture of target cells alone}} \times 100$$

Target Cells. YAC-1a Maloney murine leukaemia virus T-cell lymphoma of A strain mice: the line was maintained as suspension culture in RPMI 1640 medium with glutamine (Gibco, Grand Island, N.Y.), supplemented with 10% foetal calf serum.

Cytostatic Activity of Splenic Cells. Cytostatic activity was assayed by the method described by Tsuchiya *et al.*[9] at different intervals post-transplantation. Splenic cells (2×10^6) were cultured *in vitro* with 2×10^4 tumour cells (Walker-

256, obtained by trypsinization) in 0.2 ml RPMI 1640 medium, supplemented with 10% foetal calf serum in wells of flat-bottomed microtiter culture plates at 37°C for 72 h in 5% CO_2 atmosphere. Cells were harvested by a cell harvester. Twelve hours before radioactivity determination, in every well, 1.0 μCi tritiated thymidine was added, radioactive precursor incorporation (^{3}H-TdR) into tumoural cells being measured by a liquid scintillation counter. Cytostatic activity of splenic cells was expressed as percentage of the inhibition of ^{3}H-TdR incorporation into tumoural cells.

For calculation of the percentage inhibition of tumour growth we used the following formula:

$$\% \text{ inhibition} = \left[\frac{\begin{array}{c}1\text{-(cpm of tc + sc from}\\ \text{irrad. rats - cpm of sc}\end{array}}{\begin{array}{c}\text{only from irrad. rats)}\\ \text{cpm of (tc + norm sc)}\\ \text{- cpm norm sc}\end{array}} \right] \times 100$$

where tc = tumour cell, sc = splenic cell, irrad. = irradiated, and norm = normal.

Histology. For examination of lesions which appeared after PDT treatment, the tumours were removed from animals (from those groups of animals used for the immunological tests) after 10 days of each dose (15, 30 and 60 J/cm^2) of PDT and also the control (saline) group; tissue was fixed in buffered formalin (10%), sectioned and stained with haematoxylin-eosin.

Statistics. Data were expressed as the arithmetic mean ± the standard error (SE). The statistical significance of differences between groups was calculated by the Kruskal–Wallis test.

Results

Inhibition of Tumour Growth after Local Photodynamic Therapy

Growth curves for tumoural volumes of all six batches of animals are presented in Figure 1. In control animals (batch VI, saline injection, no PDT), tumoural volume values increased approximately 10 times, after 16 days. Growth was only slightly slower in batch (photofrin II only) and batch II (HeNe laser only). In batch III, animals exposed to photodynamic therapy at a dose of 15 J/cm^2, we noticed a relatively slower growth of tumoural volume, as compared to control batch VI. At exposure to the higher dosage of PDT (30 J/cm^2, batch IV), we noticed a slower rate of tumoural volume growth in the first 3 weeks, followed by a marked decrease in the following weeks, but without complete cure of the tumour by the 60-day mark. In contrast, in the batch V animals, treated with multiple PDT doses (6 × 60 min; 60 J/cm^2), we noticed a discrete growth of tumoural volume in the first two weeks only, followed by a complete cessation of growth.

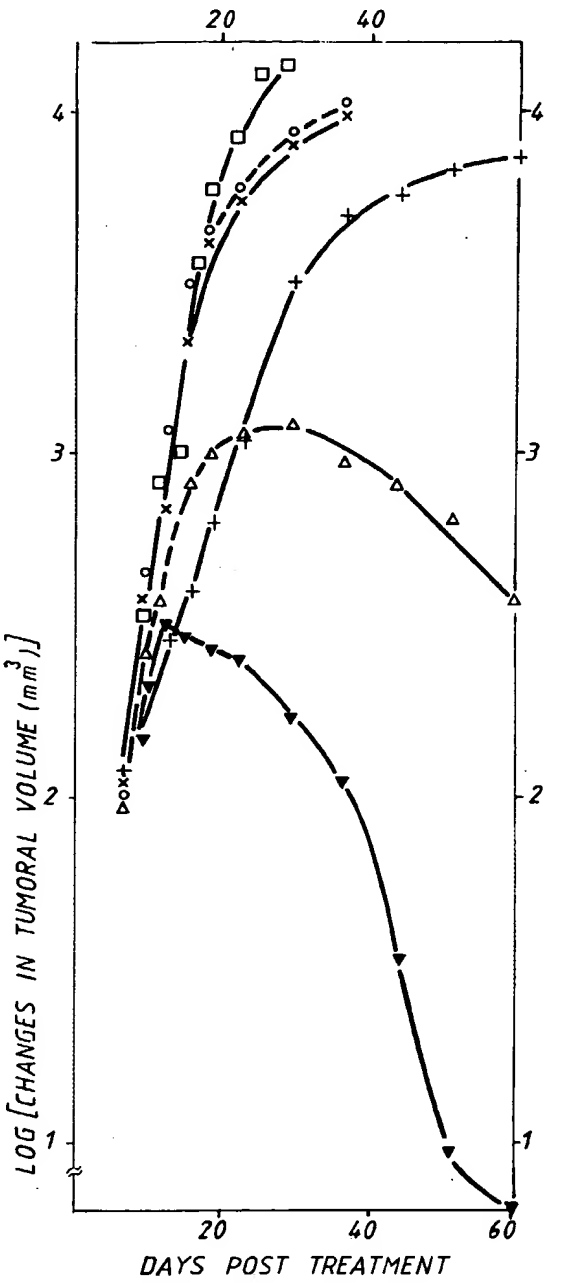

Figure 1. The change in mean tumour volume in Walker-256 carcinosarcoma-injected rats with time after photodynamic therapy. Both tumour bearing and nontumour bearing animals are included: □–□ saline-control group; ○–○ Photofrin II only; ×–× He-Ne laser only; +–+ PDT—15 J/cm^2; △–△ PDT—30 J/cm^2; ▽–▽ PDT—60 J/cm^2

Sixty days post-transplantation, examination of tumoural volume curves suggested two aspects: (a) local treatment with low PDT doses insignificantly influenced tumoural volume growth and (b) animals with photoexposure to multiple doses led to

tumoural volume diminution until a complete cure (cure rate = 31.5%) and also to an increase in the survival rates (Figures 1–3). Photofrin II alone and HeNe laser irradiation alone did not have a statistically significant influence on tumoural volume values and the animal survival rates (Figures 1 and 2).

In conclusion, in animals exposed to the optimal dose (60 J/cm²) of photodynamic therapy, the values of investigated experimental parameters were superior to the values noticed in the other batches. All these facts demonstrate the efficacy of that particular dosage rate of photodynamic therapy in Walker-256 carcinosarcoma.

Changes in the Mitogenic Response of the Host Splenic Cells by Single or Multiple PDT Doses

Cpm mean values, after lymphocyte stimulation with Con A (5 μg/ml), at 7 and 28 days post-PDT treatment, respectively, are presented in Table 1. Examining it we can notice that exposure to multiple doses produced a marked increase of radioactive precursor ^{3}H-TdR incorporation into Con A-stimulated lymphocytes, when splenic cells were cultured at 2.5 × 10^5 cells/well density; in contrast, after a single exposure to PDT (15 J/cm²) we noticed a significant reduction of mitogen response to Con A.

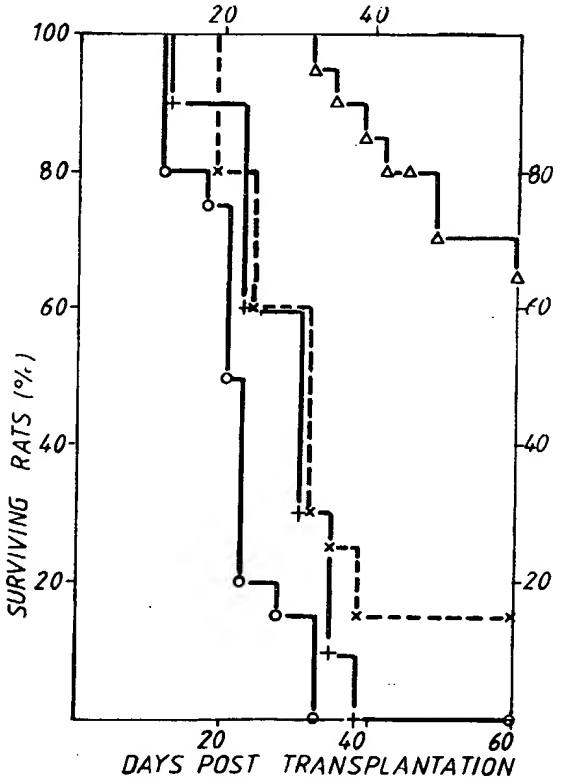

Figure 2. Therapeutic effect of photofrin II and He-Ne laser radiation on survival of rats bearing Walker-256 carcinosarcoma. Both tumour bearing and nontumour bearing animals are included: O–O saline-control group; +–+ Photofrin II; ×–× He-Ne laser; Δ–Δ PDT—60 J/cm²

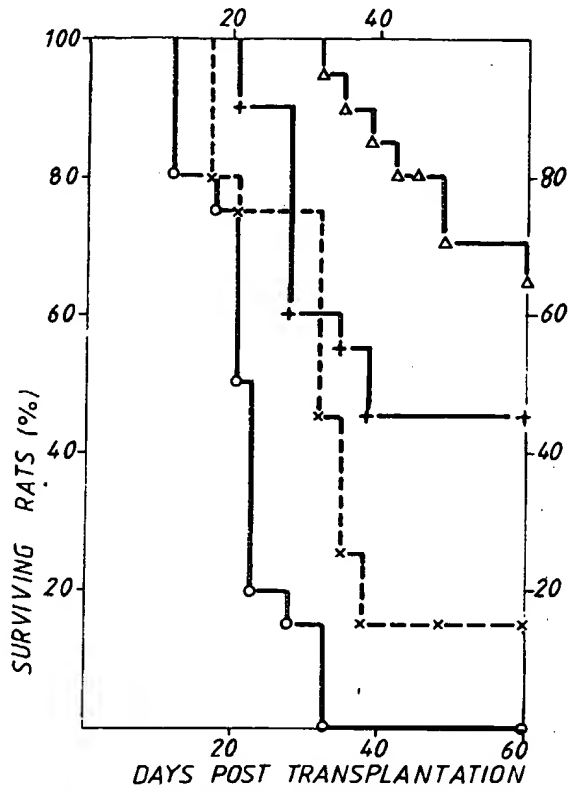

Figure 3. Therapeutic effect of different doses of radiation with photodynamic therapy on survival of rats bearing Walker-256 carcinosarcoma. Both tumour bearing and nontumour bearing animals are included: O–O saline-control group; ×–× PDT—15 J/cm²; +–+ PDT—30 J/cm²; Δ–Δ PDT—60 J/cm²

Twenty-eight days after starting PDT treatment, cpm values in batch V, after Con A stimulation, were superior to those after 7 days, especially in batches IV and V, as compared to control batches (batches I–III and VI).

From the percentage value [^{3}H]-thymidine incorporation into splenic lymphocytes, obtained from photofrin II-injected animals or HeNe laser irradiated (60 J/cm²), was inferior to that noticed in batches IV and V and approximately equal to values in batches III and VI.

Augmentation of Cytotoxic Activity After Photodynamic Therapy. The experiments presented in Table 2 aimed to determine the capacity of photodynamic therapy to stimulate cytotoxic activity of splenic lymphocytes and to lyse target YAC-1 cells.

Results demonstrated that after a single exposure to PDT (15 J/cm²), cytotoxic activity was non-specifically stimulated. Maximal cytolysis against target YAC-1 cells was noticed in splenic lymphocytes from multiple PDT doses treated animals (batches IV and V). In contrast, i.p. injection of

Table 1. Response of splenic lymphocytes to Con A in animals with Walker-256 carcinosarcoma locally treated with different doses of photodynamic treatment

Batch*	Treatment (J/cm²)	cpm after Con A stimulation: 7 days after treatment	cpm after Con A stimulation: 28 days after treatment
I	Photofrin II	18 962 ± 1876	20 835 ± 1569
II	He-Ne laser	19 954 ± 1663	23 114 ± 2043
III	PDT—15	20 139 ± 1589	21 412 ± 2956
IV	PDT—30	23 746 ± 1983	28 438 ± 3097
V	PDT—60	27 994 ± 3067	34 724 ± 2688
VI	Control (saline)	19 365 ± 2365	22 873 ± 1724

*Healthy control animals: 32 965 ± 1146.

Table 2. Dependence of cytotoxic activity of splenic effector cells from animals with Walker-256 carcinosarcoma and dose exposure to photodynamic therapy

Batch†	Treatment (J/cm²)	Percentage of cytotoxicity against target YAC-1 cells*: 7 days after treatment	Percentage of cytotoxicity against target YAC-1 cells*: 28 days after treatment
I	Photofrin II	11.3 ± 0.3	10.4 ± 0.7
II	He-Ne laser	12.1 ± 0.5	14.7 ± 0.8
III	PDT—15	13.6 ± 0.4	15.9 ± 0.6
IV	PDT—30	19.2 ± 0.8	22.5 ± 1.4
V	PDT—60	24.3 ± 2.1	29.8 ± 2.6
VI	Control (saline)	11.8 ± 0.5	10.9 ± 0.6

*Splenic cells from Wistar inbred rats with Walker-256 carcinosarcoma were assayed at 7 and 28 days respectively for cytotoxic activity against YAC-1 target cells at a 200:1 effector/target cell ratio.
†Healthy control animals: 27.9 ± 1.9.

the photosensitizing agent alone or a single exposure to HeNe laser alone were not capable of significantly increasing effector cells' cytotoxic activity.

The results suggest two aspects: (a) PDT local irradiation increases cytotoxic activity against target cells and (b) this activity takes place in a dose-dependent manner. Spontaneous release of the isotope from target cells was less than 12.5% during the test.

Cytostatic Activity of Splenic Cells from Tumour Photodynamic Therapy. In splenic cells from control animals (saline) and in those treated with a single PDT dose (15 J/cm², batch III) we noticed an increase of cytostatic activity with a peak within 6–8 days post-transplantation, followed by a decrease nearly up to baseline.

In contrast, in splenic cells isolated from PDT multiple dose-tested animals (batches IV and V), we noticed a marked increase of cytostatic activity (inhibition of radioactive precursor [3H-TdR] incorporation into carcinomatous cells), starting in day 6 and maintaining high values during treatment, as can be seen in Figure 4.

Histopathology. The following morphological modifications were noticed in the different PDT (15–60 J/cm²) treated Walker-256 carcinosarcoma: in samples obtained from control batch VI (saline), we noticed tumoural proliferation with an average of small cells, oval or rounded, presenting nuclei with rich chromatin (hyperchromatic), comparatively reduced cytoplasm, weak basophil; here and there, there were larger cells with budding or multiple nuclei, presenting characteristic atypia for tumour cells. The cells did not present adhesion, appearing individualized; between cells, spaces could be seen where red cells and proteinform type granular structures were noticed; also individualized blood-dilated or haemorrhaging capillaries in tumoural mass, could be seen. In the tumour mass, we noticed unequal zones of necrosis, leading to the conclusion the tumour was a weakly differentiated tumour of sarcomatoid type (Figure 5).

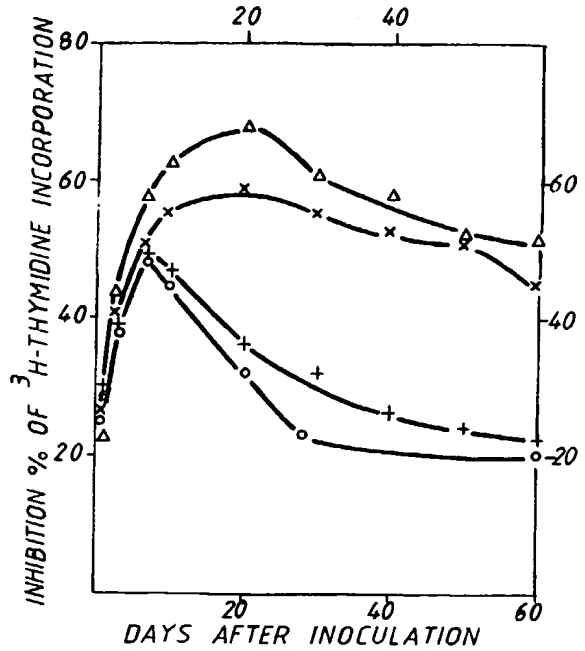

Figure 4. Change of cytostatic activity of spleen cells after tumour photodynamic therapy. O–O saline-control group; +–+ PDT—15 J/cm²; ×–× PDT—30 J/cm²; △–△ PDT—60 J/cm²

Figure 6 presents a fragment of epidermis and dermis without significant modifications. The sample was obtained from animals with Walker tumours treated with a single PDT dose (15 J/cm²) and sacrificed after 10 days. In the prevelant layer, under the striated muscular tissue, an intricate arrangement of small-sized tumoural cells with numerous capillary neoformation and lympho-fibrocytic reactive elements may be noticed; in other ones the histological picture presents a mixomatous conjunctival tissue, with cells having star-like prolongations, near zones with less oedematous aspect, the cells acquiring a prolonged aspect, some of them being of fibrocytic type and others showing tumoural characteristics in volume, size, and nuclear hyperchromatosis: these are typical aspects of fibrosarcoma.

In Figure 7, we present a cutaneous fragment with epidermis and hypodermis, harvested 10 days after last PDT exposure (30 J/cm²); it does not show significant histopathological modifications. At the dermal level, we detect granulation tissue, rich in cells and vascularized, with an intricate arrangement of tumour cells; distinguishing them from reactive elements within stromal reaction process is difficult in certain zones, except the deep peripheral zones near muscular tissue where these tumoural elements appear clear and individualized, having a more prolonged form, and mostly small in size; here and there larger cells appear with bigger and hyperchromatic nuclei. This enabled us to stress the richness of the vasculo-conjunctival reaction layer which determines changes into the cytological aspect

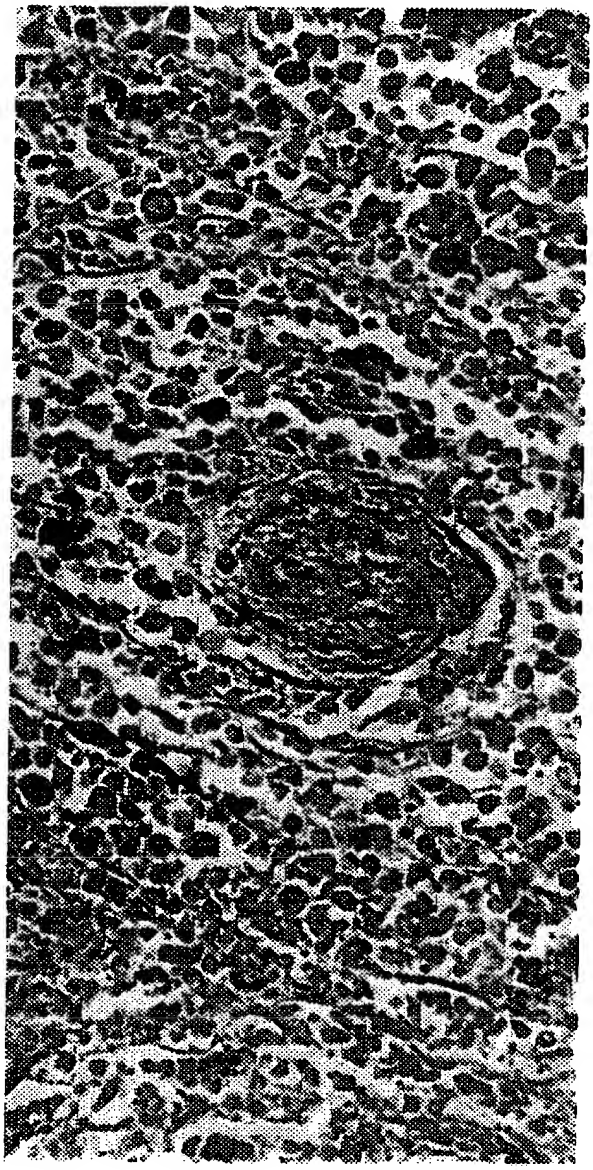

Figure 5. Control rat. Histological aspect of Walker-256 carcinosarcoma (not treated). Hematoxylin-eosin staining. × 572

of the tumour, but making difficult any identification, except at the peripheral limited zones lacking in reactive granulation tissue.

Samples obtained from batch V animals with 60 J/cm²-treated tumours (Figure 8) showed a tegument without significant modifications and tumoural proliferation; under the striated muscular layer there are fusiform, round, oval infiltrates, separated by local oedema; cells with tumoural characteristics are isolated and rare (more varied volume, nuclei with hyperchromasia, atypic). Figure 9 presents a similar image where tumoural infiltrates with small, round cells appear under the striated muscular layer. At

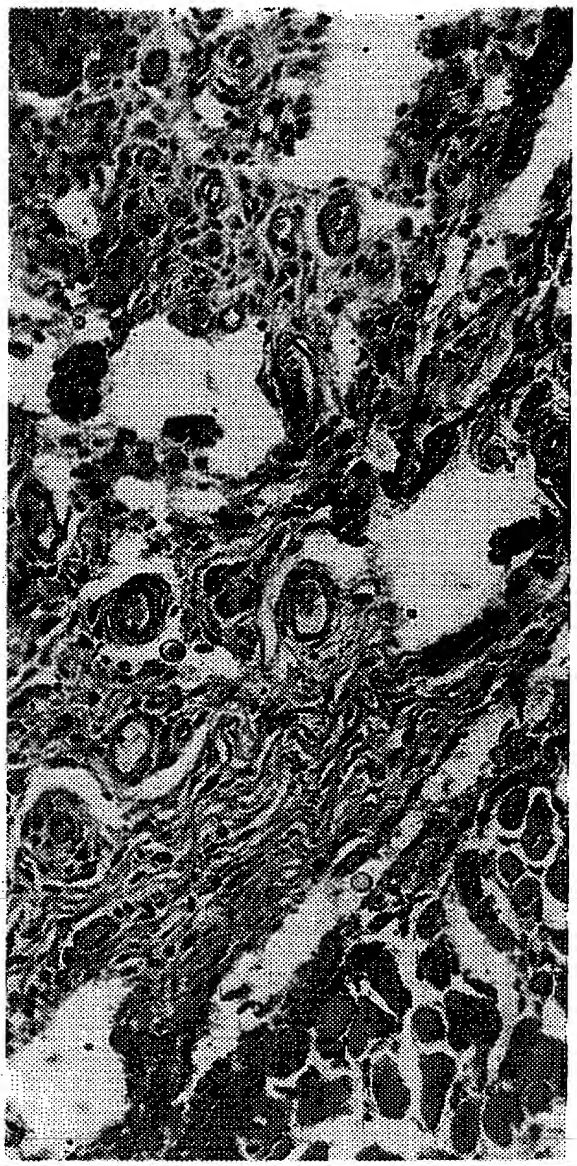

Figure 6. Section through dermis, hypodermis and muscular tissue. Sample removed from animals with Walker-256 carcinosarcoma after single exposure to photodynamic therapy (15 J/cm²). Hematoxylin-eosin staining. × 572

Figure 7. Skin fragment removed from rats with PDT-treated Walker-256 tumour (30 J/cm²). Hematoxylin-eosin staining. × 572

various levels we notice vascular modifications of the obliterating endovascularitis type.

From the same batch (V), we obtained the section seen in Figure 10 showing a cutaneous fragment with normal-appearing epidermis and dermis. Pilous folliculi present rate mononuclear, lymphocytic infiltrations. An inflammatory reaction with a lymphocytic component is seen together with fibrocytic elements having conjunctival fibres; also arterioles and venules with endovascularitis processes showing obliterating tendency are present. No tumoural proliferation is detected.

Discussion

In the present work and in previous ones[13,14] we have shown that photodynamic treatment reduces mortality, volume and incidence of tumour. The maximal period of 2 weeks (7–21 days post-transplantation) was chosen because we had noticed, in previous studies, a significant response of cell-mediated antitumoural immune system[15] within this time.

The values of the experimental parameters under study, during 53 days from the start of photodynamic treatment, amplified the variation of the immune

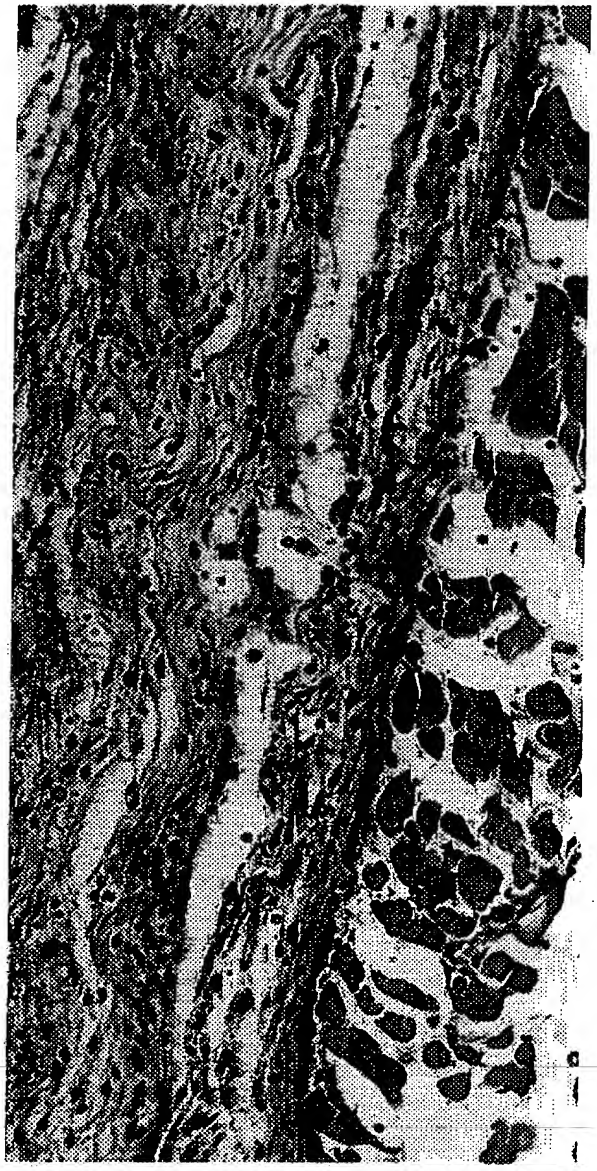

Figure 8. Skin fragment removed from animals with PDT-treated Walker-256 tumour (60 J/cm²). Hematoxylin-eosin staining. × 256

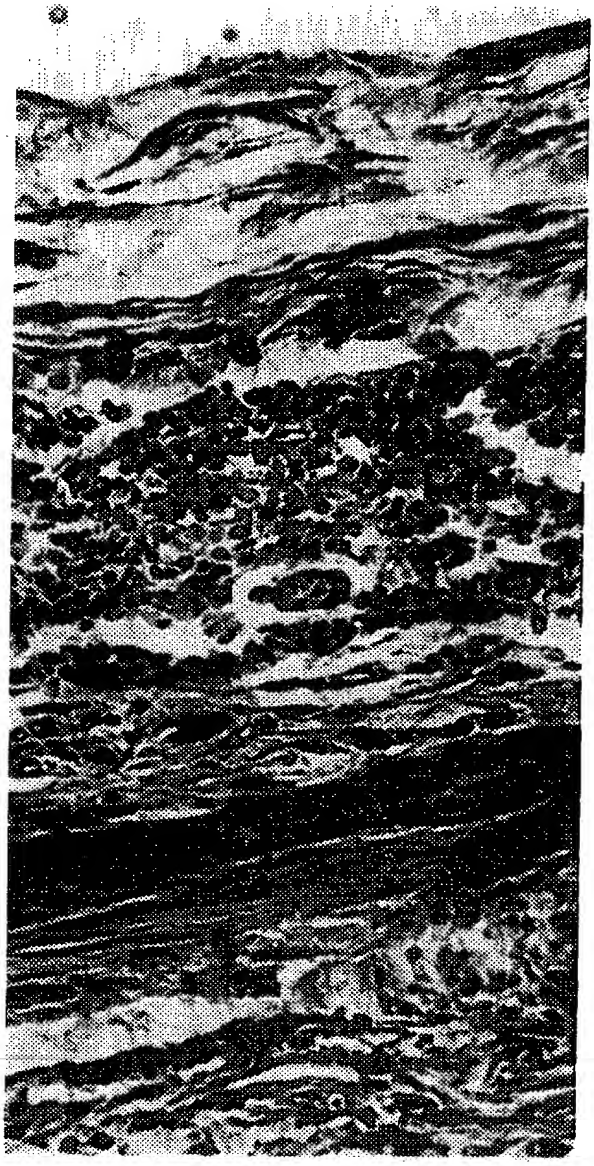

Figure 9. Section through dermis, hypodermis and muscular tissue, removed from rats with Walker-256 tumour, after exposure to multiple PDT dose (60 J/cm²). Hematoxylin-eosin staining. × 572

response between the different batches, due to each treatment type.

Experimental results showed that repeated exposure to photodynamic therapy led to stimulation and maintenance of high values of cell-mediated antitumoural activity for 28 days post-treatment (Tables 1 and 2 and Figure 4). In contrast, after a single photofrin II treatment or HeNe laser exposure, a significant increase of cell-mediated antitumoural immunity was not detected (Tables 1 and 2).

For the appearance of these beneficial effects, we could provide several explanations: (a) increase of cytotoxic lymphoid cells antitumoural activity; (b) increase in cytotoxic activity during a relatively long period (6–10 days), in such a manner that cytotoxic activity would contribute to elimination of tumoural cells, which aspect is relevant in our experimental design; (c) photodynamic treatment increased activity of lymphocytes and of other nonirradiated regions (liver, lung, blood; experiments in progress).

In support of our results, we can mention data provided by other researchers, showing that tumoural cell survival is strongly inhibited by host immune response after local irradiation.[16] Host cell-mentioned antitumoural immunity was demonstrated to be induced or increased by local irradiation

Figure 10. Section through dermis, hypodermis and muscular tissue, removed from rats with Walker-256 tumour, after exposure to multiple PDT dose (60 J/cm^2). Hematoxylin-eosin staining. × 130

of the tumour and also splenic cells of such animals were proved to be capable of inhibition of the growth of tumoural cells.[9,10] Recently, it has been suggested that cell-mediated antitumoural immunity could depend on cooperation between non-killer T-cells and macrophages.[10]

Our experimental observations are in agreement with works of some authors,[8–10,17] sustaining the appearance of an increased immune response 7 or more days after local irradiation, but are contradicted by other authors who indicate such an increase in only a single day after irradiation.[7] These discrepancies could be explained by different methods used for immunological activity evaluation.

At present, we do not know why cell-mediated antitumoural specific activity increases after tumour exposure to multiple PDT doses. It was suggested that it could be induced by modifications of surface tumoural antigens after irradiation[18] or because of alterations in suppressor–effector T-cells ratio, as in the case of surgical excised tumours.[19]

Histopathological modifications noticed 10 days after exposure to singular or multiple PDT doses are in full agreement with other authors' observations referring to possible mechanisms that could act in photodynamic therapy. Among most important mechanisms we emphasize: (a) direct effects on malignant cells induced by oxygen presence as single oxygen;[20] (b) superoxide production;[21] (c) ischemia resulting from vascular lesions which could be the main cause of tumour destruction during photodynamic therapy;[22–26] (d) potentiation of cell-mediated antitumoural immune response after photoexposure to multiple doses of PDT.[15]

Data presented in this work demonstrate that exposure to local photodynamic treatment in multiple doses stimulates cell-mediated antitumoural activity, produces modifications in tumour histological structure, increases survival rate and reduces tumoural incidence in Walker-256 carcinosarcoma in the rat model.

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L5 ANSWER 8 OF 8 CAPLUS COPYRIGHT 2002 ACS
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TITLE: Method for isolating phage or plasmid DNA from bacteria
INVENTOR(S): Masafumi, Murata; Munechika, Sakaba
PATENT ASSIGNEE(S): Seiko Instruments, Inc., Japan
SOURCE: Eur. Pat. Appl., 5 pp.
CODEN: EPXXDW
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LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
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EP 240191	A3	19891025		
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JP 1986-77853 19860404

AB A method is presented for isolating DNA from a virus or bacterium that consists of the following steps: (1) isolating the virus or a bacterium which has the satellite DNA; (2) reaction with a **protein denaturing** agent, such as **proteinase K** or SDS, to denature proteins assocd. with the DNA; (3) adding twice by vol. of alc. to the reaction mixt. to ppt. the DNA; (4) passing this mixt. through a sepn. column to retain the pptd. DNA; (5) passing an aq. alc. soln. having a high alc. concn. through the column to remove impurities; and (5) eluting the DNA from the column with an aq. soln. free from alc. The method was used to isolate DNA from M13 and .lambda. phages contained in Escherichia coli and for isolating plasmid pBR322 from E. coli.

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1. Proceedings of SPIE-The international society for optical engineering:
   1999, Vol. 3601, (Laser-Tissue Interaction X), pp. 75-81
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Clinical study of biological response modifiers as maintenance therapy for hepatocellular carcinoma

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Abstract. We conducted a randomized, controlled trial comparing 5-fluorouracil (5-FU) with or without biological response modifiers (BRMs) as a maintenance therapy for hepatocellular carcinoma (HCC) after treatment with percutaneous ethanol injection (PEI), transcatheter arterial embolization (TAE) or arterial infusion of antitumor agents (AI). A total of 58 cases of HCC were classified into 4 groups as follows: group I, PSK with 5-FU (n = 15); group II, lentinan with 5-FU (n = 15); group III, OK-432 with 5-FU (n = 12); and group IV, 5-FU alone as the control (n = 16). The mean survival time, mortality rate, time to progression, and T_4/T_8 ratio of lymphocytes in the peripheral blood were compared among the four groups. There was no significant difference in the background factors among the groups. In group I, the T_4/T_8 ratio of lymphocytes was reduced after the therapy. No significant difference was found among the groups in terms of the mean survival time, mortality rate, or time to progression. PEI for initial therapy was superior to the other therapies in terms of the mean survival time and mortality rate. These results suggest that the addition of BRM to maintenance therapy with 5-FU exerts no prognostic benefit on HCC patients treated with PEI, TAE, or AI.

Introduction

Percutaneous ethanol injection (PEI), transcatheter arterial embolization (TAE), and arterial infusion of antitumor agents (AI) are being established as effective therapies for patients with inoperative hepatocellular carcinoma (HCC) [1, 4, 9]. However, these methods are not suitable for maintenance therapy after tumor reduction or a relative decrease in size because, depending on the patient's condition, they cannot be performed on a long-term basis.

There have been quite a few reports concerning the treatment of HCC with biological response modifiers (BRMs), and the effectiveness of BRMs for HCC patients has not been established. We conducted a randomized controlled trial comparing 5-fluorouracil (5-FU, tablet) with or without BRMs as maintenance therapy for HCC after treatment with PEI, TAE, or AI.

Materials and methods

1. Research period and institution. From June 1987 to October 1992, a joint study was conducted in our department and the branch institutions shown in Table 1.

2. Patients. Patients with HCC who had been treated with PEI, TAE, or AI and showed a therapeutic effect of at least "no change" (NC) as evaluated by the criteria of the Japan Society for Cancer Therapy were eligible for this study. The entry requirements included an expected survival of more than 3 months, the absence of any serious cardiac or renal problem, the absence of hypersensitivity to OK-432, a minimal white blood cell count of 2,000/mcl, a minimal platelet count of 4×10^4/mcl, and a minimal hemoglobin concentration of 8 g/dl. In all cases, informed consent was obtained from the patient or a family member.

3. Treatment. The method of administration of BRMs was as follows. All patients were given 5-FU orally at 100–150 mg every day. They

Table 1. Cooperating institutions

1. First Department of Internal Medicine, Hirosaki University School of Medicine
2. Department of Internal Medicine, Kuroishi Municipal Hospital
3. Department of Internal Medicine, Kensei Hospital
4. Department of Gastroenterology, Aomori Prefectural Central Hospital
5. Second Department of Internal Medicine, Aomori Rohsai Hospital
6. Department of Internal Medicine, Misawa Municipal Hospital

Work presented at the Third International Symposium on Treatment of Liver Cancer, Seoul, Korea, 12–13 February 1993

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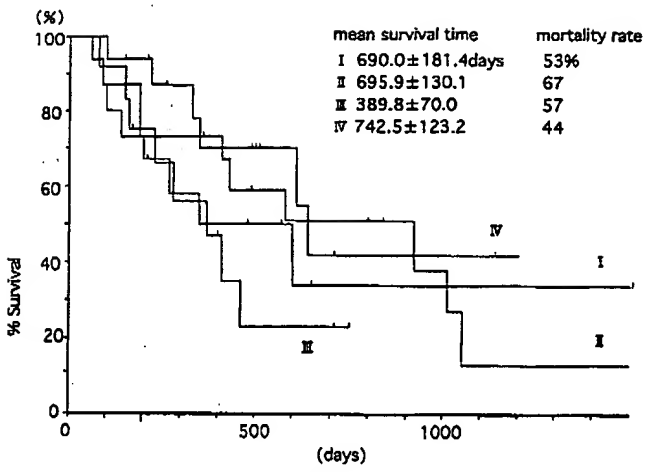

Fig. 1. Survival by treatment group

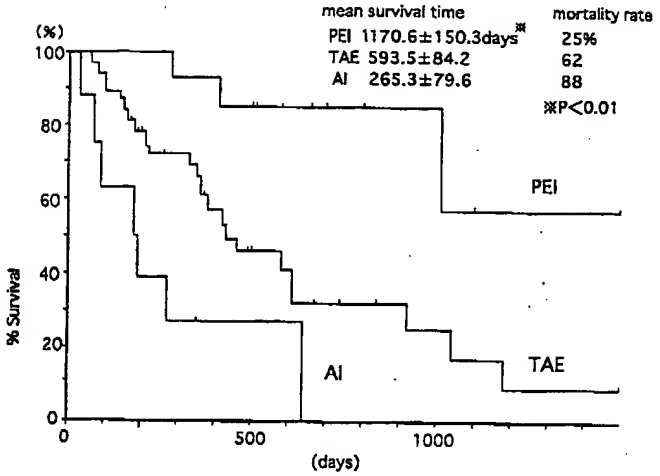

Fig. 2. Survival as a function of the initial therapy

Table 2. Patients' characteristics

Characteristic	Group				Total
	I	II	III	IV	
No. of patients	15	15	12	16	58
Sex:					
M	11	12	8	9	40
F	4	3	4	7	18
Age (mean, years)	58	62	65	66	62
Child's classification:					
A	7	8	4	11	30
B	3	7	6	5	21
C	5	0	2	0	7
Initial therapy:					
PEI	7	10	8	13	38
TAE	3	3	4	2	12
AI	5	2	0	1	8

Table 3. Time to progression in each treatment group

Group	Time to progression (days)	Significance level
I	250,2 ± 153.7	NS
II	344.1 ± 266.3	NS
III	252.2 ± 199.3	NS
IV	299.3 ± 209.5	NS

NS, Not significant

Table 4. Change in the T_4/T_8 ratio after BRM

Group	Before BRM	After BRM
I	2.33 ± 1.63	1.91 ± 1.25*
II	2.23 ± 1.36	2.20 ± 1.21
III	2.12 ± 1.13	2.40 ± 1.39
IV	1.99 ± 0.50	2.07 ± 0.69

* $P < 0.05$

were allocated into four groups. Group I was given 3 g PSK orally every day for 7 consecutive days every 2 weeks. Group II was intravenously injected with 2 mg of lentinan (LTN) once every week. In group III, OK-432 was subcutaneously injected once per week, and the dose was gradually increased from 0.2 KE to 5 KE, depending on the patient's condition. Group IV was given a daily dose of only 100–150 mg 5-FU orally (active control). Allocation was done randomly by the envelope method. Observation of the general condition, laboratory tests, tumor marker tests, and various image diagnoses were performed once a month, in principle. The mean survival time, mortality rate, time to progression, and T_4/T_8 ratio of lymphocytes in the peripheral blood were also determined.

Results

1. Enrolled cases

A total of 65 patients were entered into this study, including 16 in group I, 16 in group II, 17 in group III, and 16 in group IV. In all, 3 ineligible patients were completely excluded from the analyses. The remaining 62 patients consisted of 58 protocol-adhering patients and 4 patients who violated the protocol because of side effects due to BRMs. Finally, 58 of the 65 patients enrolled (89%) were included in the analyses, including 15 in group I, 15 in group II, 12 in group III, and 16 in group IV.

2. Analyzed cases

Table 2 presents the background characteristics of the patients; there was no significant difference in sex, average age, Child's classification, or previous treatments. However, five of the seven Child's C cases were in group I and two were in group III, whereas five of the eight AI cases were in group I. Thus, group I showed a tendency to include more cases with rather deteriorated reserve liver function.

The survival rate of each group was determined by the Kaplan-Meier method (Fig. 1). The mean survival time was 690.0 ± 181.4 days in group I, 695.9 ± 130.1 days in group II, 389.8 ± 70.0 days in group III, and 742.5 ± 123.2 days in group IV, with no significant difference being found among them. The mortality rate was 53% in group I, 67% in group II, 57% in group III, and 44%

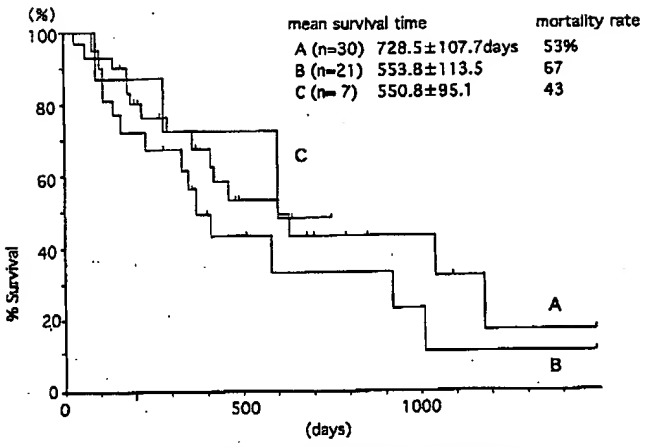

Fig. 3. Survival as a function of the Child's classification

Table 5. Mean survival time and mortality rate as a function of tumor size

Size (cm)	Mean survival time (days)	Mortality rate (%)
≦2 (n = 18)	759.8 ± 109.8	46
2~5 (n = 29)	687.3 ± 112.1	52
>5 (n = 16)	509.5 ± 106.4	75

in group IV. The mean time to progression was 250.2 ± 153.7 days in group I, 344.1 ± 266.3 days in group II, 252.2 ± 199.3 days in group III, and 299.3 ± 209.5 days in group IV, and the differences among the groups were not significant (Table 3). The T_4/T_8 ratio of lymphocytes in the peripheral blood did not change after the BRM therapy for more than 3 months except in group I, where the ratio decreased after the therapy (Table 4).

A study was made of the factors affecting the patients' survival. The mean survival time compared as a function of the previous therapy was 1170.6 ± 150.3 days for PEI, 593.5 ± 84.2 days for TAE, and 265.3 ± 79.6 days for AI, and the survival rate was significantly higher for PEI than for the other two groups (Fig. 2). The mortality rate was 25% for PEI, 62% for TAE, and 88% for AI, showing the same favorable tendency for PEI. The mean survival time compared as a function of the Child's classification was 728.5 ± 107.7 days for Child's A, 553.8 ± 113.5 days for Child's B, and 550.8 ± 95.1 days for Child's C, with no clear difference being found among the Child's classification groups (Fig. 3). The tumors were divided into three groups on the basis of the diameter being 2 cm or less, 5 cm or less, and more than 5 cm, and the average survival and mortality were compared. No clear difference in mean survival time was observed among the three groups (Table 5).

Discussion

Recently, the mortality due to HCC has been increasing in Japan, and various therapies are being tried against it. At present, PEI and TAE are considered to be effective and are frequently used for inoperable HCC. However, these methods are not suitble for long-term maintenance therapy and should be used once or a limited number of times for remission induction. For maintenance, the use of oral drugs other than 5-FU or UFT is rare, and few studies have been conducted on other therapeutic methods [3, 5].

BRMs are considered to act as enhancers of the antitumor effects of radiation and chemotherapies, thereby preventing the recurrence or metastasis of cancer after surgery [7]. It has been postulated that in patients with HCC, the immunity is generally lowered due to the cancer itself and to cirrhosis, which is often a complication of HCC in Japan [6].

Under these circumstances, we concluded that it was necessary to carry out a randomized control study of the various effects of BRMs as maintenance therapy after the performance of PEI, TAE, or AI. We thus started a cooperative study at several research institutions.

Three kinds of BRM, i.e., PSK, LTN, and OK-432, which are generally used for other solid cancers, were selected and compared with one another. In the present study, the BRM therapy was not effective since the results did not show any clear difference from those obtained in the control group. Hirai et al. [2] reported that concurrent use of carmofur or tegafur with OK-432 for maintenance therapy after TAE was effective. However, some difference was demonstrated in our study, in which the previous therapy was different from that used by Hirai et al. Furthermore, although no significant difference was found, there may have been some influence arising from the observations that Child's C cases existed only in group I and that PEI was performed more often in the control group. Takezaki et al. [8] studied the concurrent use of 5-FU or UFT with LTN in patients with HCC treated by TAE or AI, and their results concurred with ours. We believe that it is necessary to carry out a study involving a greater number of patients to search for a more effective therapeutic method during the period of clinical follow-up after PEI, TAE, or AI.

The previous therapy has a strong influence on the response of inoperable HCC, i.e., in cases previously treated by PEI, the mean survival time was extended and the mortality was lowered. No clear difference was found in the comparison of the mean survival time and the mortality rate among the groups as a function of the Child's classification or the tumor diameter. These results suggest that a longer survival period can be expected at present if the HCC can be discovered when PEI is applicable.

The reason for starting the present cooperative study was the hope of discovering a better therapeutic method for use during the period of maintenance after PEI, TAE, or AI therapy. The authors think that further study is needed in the future regarding the kinds of BRMs, their dosage, administration frequency, and other factors so as to achieve better maintenance therapy for HCC after induction of remission.

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COMPLETED

Cavitation thresholds in the rabbit retina pigmented epithelium

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ABSTRACT

We performed measurements to examine retinal injury from laser pulses in the sub-nanosecond time regime. Both *ex-vivo* porcine and Dutch belted rabbit retinal pigment epithelium (RPE)[¥] models were used in conjunction with time resolved imaging to observe cavitation bubble formation. Included in this study are 3 ps, 300 fs, and 100 fs pulses at a wavelength of 580 nm, as well as 70 ps and 5 ns pulses at 532 nm. Threshold values varied between 37 mJ/cm^2 and 50 mJ/cm^2 across the range of pulse widths. Following laser irradiation cell viability was checked using a fluorescent dye marker (calcein). Our current results are compared to an earlier investigation using the artificial retina model.

1. INTRODUCTION

Damage induced by ultrashort laser pulses incident on the eye has been of great concern in recent years due to the commercial availability of sub-nanosecond laser systems. Research efforts have concentrated in two main areas: (1) determining threshold values for a detectable damage endpoint, and (2) determining the mechanism(s) responsible for damage at the different pulse widths. Our experiments have addressed both of these issues through targeting RPE cells for laser impingement and discussing the most plausible mechanism(s) in terms of known non-linear phenomena.

The standard technique for ascribing a threshold value is by empirically establishing that energy level which will produce damage in the target 50% of the time (commonly know as the ED_{50} level). Typically live animal models have been used for retinal minimum visible lesion (MVL) experiments. The current project is an *ex vivo* procedure where recently inoculated rabbit and porcine eyes were dissected to expose the RPE layer, and irradiated with ultrashort laser pulses. We chose to use both procine and rabbit tissues to compare to previous studies, and to extend the validity of these experiments to several models. The advantages in using both rabbit and porcine RPE are (1) that investigations on two separate species are performed utilizing similar conditions and criteria, and (2) porcine eyes more closely mimic structurally the human eye.

[¥] The animals involved in this study were procured, maintained, and used in accordance with the Federal Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources - National Research Council.

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All viability assays in this work were performed with calcein, a member of the fluorescein family of dye markers. Living cells will metabolize the dye, and when stimulated by the 488.0 nm line from an argon ion laser, will fluoresce. Long pass filters were used to block the exciting wavelength. We examined two different scenarios: tagging the cells before laser incident, and applying the assay after irradiation had occurred. In each case the result was the same; the cells remained alive for sub-threshold exposure, but lost viability following even a single exposure at an energy sufficient to cause cavitation[3].

2. EXPERIMENT

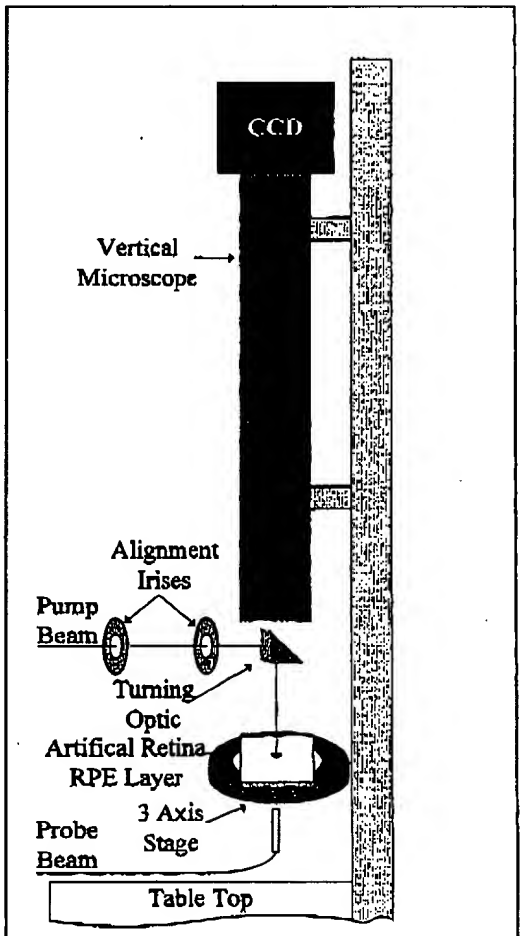


Figure 1. Side view of experimental setup for observing bubble formation from irradiated RPE layer.

Figure 1 illustrates the vertical microscope imaging system. The ultrashort laser system contains a mode-locked (82 MHz) pulse compressed, frequency doubled Nd:YAG laser used to excite a dye laser that produces a 100-fs, 580 nm beam. This beam is then amplified by a three-stage pulse dye amplifier (PDA), with the PDA being pumped by a seeded, frequency doubled Nd:YAG regenerative amplifier. Upon exiting the PDA the laser light is

directed through a spatial filter so that a Gaussian beam profile is achieved. The laser system produces 100 fs, 300 fs and 3 ps pulses at 580 nm; 80 ps and 3 ns pulses at 532 nm; and 80 ps and 5 ns pulses at 1064 nm. In the case of the vertical microscope the focus of the objective was fixed and the sample was positioned using three micrometer driven stages. The objective itself has a long working distance and a large numerical aperture. The ultimate resolution of the objective is 1 μm according to the manufacturer. Also, the strobe illumination was brought in beneath the target, and was timed to be 1 μs after the pump pulse delivery. The calcein assay was diluted 1000:1 in phosphate buffered saline (PBS) and applied to the tissue approximately 15 minutes prior to fluorescence image acquisition. In this way RPE cell viability both before and after laser irradiation was confirmed.

3. RESULTS

Our *ex vivo* investigations indicate a near constant fluence of approximately 40 mJ/cm^2 across the range of pulse widths in the visible regime. These results are summarized graphically in Figure 2. This behavior is consistant with findings previously reported by our lab concerning damage thresholds in the artificial retina[2], namely that the water path is an important factor for determining the eventual *in vivo* retinal damage threshold. We also performed viability tests to observe the RPE cells response to laser exposure of varying energies. Figure 3a shows a pre-irradiation RPE fluorescence image, demonstrating that the cells are still alive. Figure 3b is the fluorescence picture of the targeted cells about one minute post laser irradiation. Clearly evident is that while the RPE cells may not experience pronounced morphological change in response to laser insult, cell death occurs for fluences at or slightly above the ED_{50} level. Other authors have reported similar trends[3].

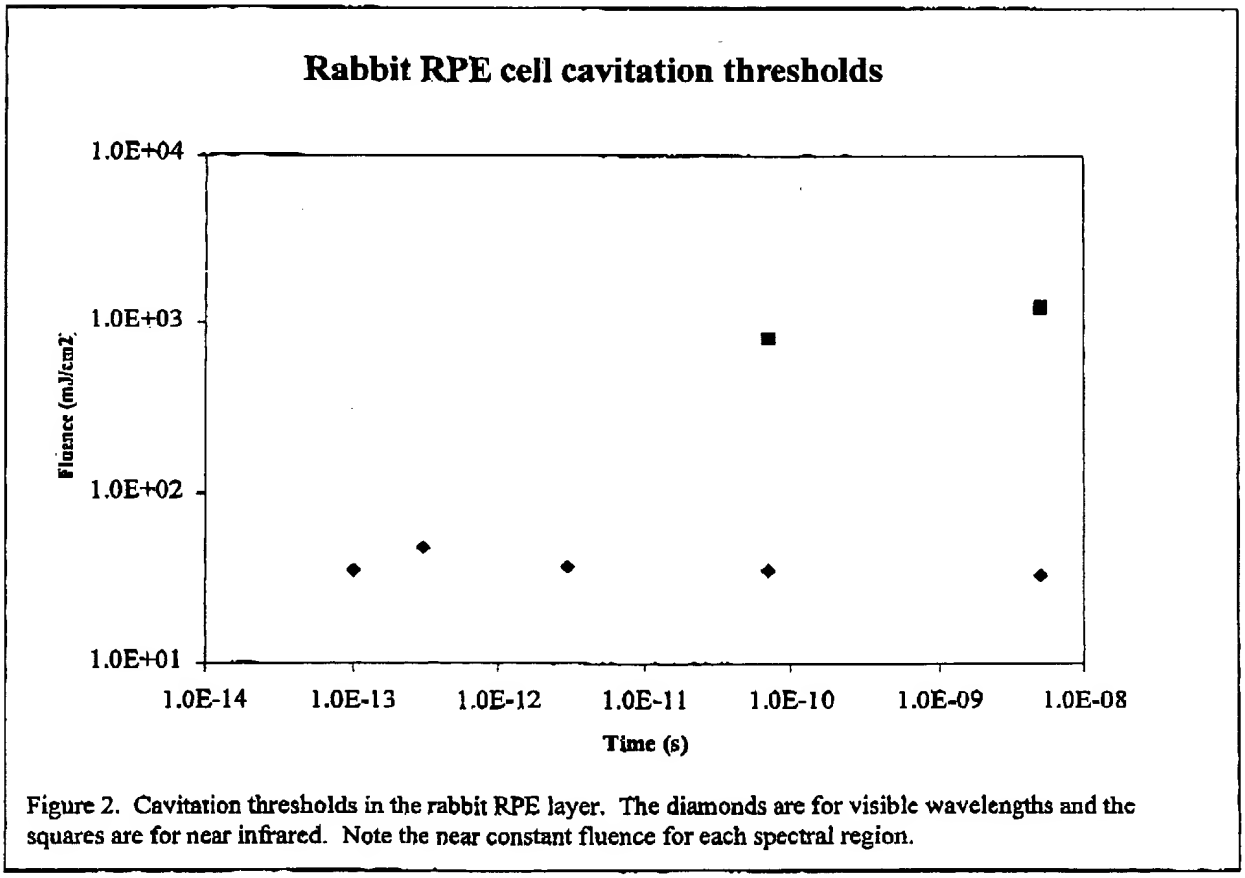
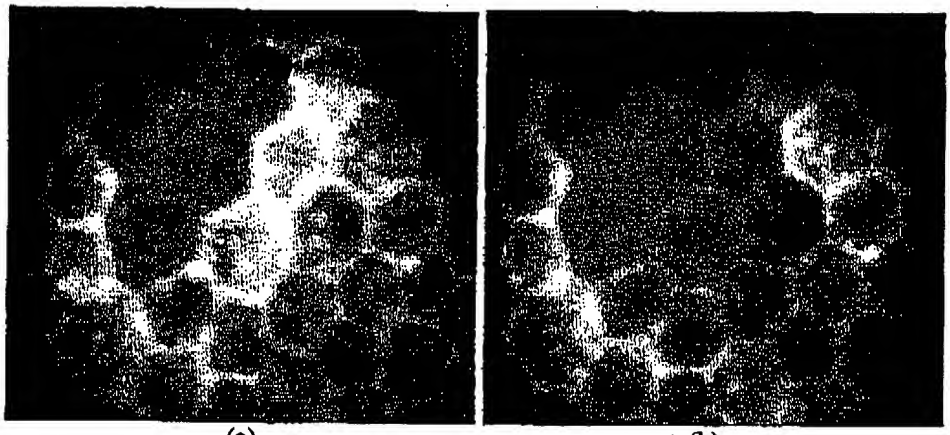


Figure 2. Cavitation thresholds in the rabbit RPE layer. The diamonds are for visible wavelengths and the squares are for near infrared. Note the near constant fluence for each spectral region.



(a) (b)

Figure 3. RPE viability assay using calcein. The picture on the left is before laser irradiation, and the image on the right is after laser pulse delivery.

4. DISCUSSION AND SUMMARY

The trends shown in Fig. 2, i.e. a near constant fluence for both NIR and visible wavelengths, reinforces our assertion that MVL threshold values are sensitive to the laser propagation path. Note that for longer pulse widths, such as the nanosecond regime, the vertical microscope *ex vivo* RPE measurements and the *in vivo* MVL ED_{50} studies yield similar results. However, as the pulse width decreases and non-linear processes like laser induced breakdown (LIB) become manifest, the living biological system responds by requiring a reduced amount of energy to cause damage. The current *ex vivo* work maintained only a thin layer of phosphate buffered saline (PBS) solution approximately 1 mm in depth above the RPE cells to prevent them from drying out during the experiment. The lack of intermediate media has the effect of prohibiting any non-linear processes from developing, and hence the same amount of energy is needed for cavitation bubble formation across the nanosecond to femtosecond range. To conclude, we measured cavitation bubble formation thresholds in rabbit RPE and found the ED_{50} to be about 1 J/cm^2 for the near infrared, and about 40 mJ/cm^2 for the visible portion of the spectrum.

5. ACKNOWLEDGMENTS

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Histopathology of Ultrashort Pulsed Laser Retinal Damage: Changing Retinal Pathology With Variation in Spot-Size for Near-Infrared Laser Lesions

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ABSTRACT

Purpose: We wish to identify the change in extent of retinal tissue injury due to varying the spot size at the retina of ultrashort laser pulses.

Methods: We compared the effects of delivery of near infrared (1060 nm) single laser pulses to an 800 micron diameter retinal spot to previously reported laser retinal effects. We examined macular lesions 24 hours after delivery of near-infrared (1060 nm wavelength) ultrashort laser to 804 micron spot-size, using fundus examination, fundus photographs and fluorescein angiograms. Using light microscopy, we examined sections of these lesions obtained 24 hours after laser delivery. The degree of retinal damage was compared to our data published previously by using a modified version of our previous grading scale.[1]

Results: The 150 fs near infrared, large spot laser lesions were remarkable in their clinical and pathological appearance. The lesions, rather than centering on a single focal spot of pallor as typically seen in pulsed laser lesions of the retina, demonstrated a spotted pattern of multiple focal lesions across the area of laser delivery. There was also choroidal damage in several eyes but the Bruch's membrane remained intact. Although there was choroidal damage in the 150 fs near infrared wavelength small spot laser lesions there was not significant thermal spread. The small spot ultrashort visible wavelength showed no significant thermal spread and no choroidal damage.

Conclusions: Larger spot-size demonstrated a broader area of damage than that of the smaller spot-size and different choroidal effect when compared to smaller sized lesions.

Keywords: choroidal damage, histopathology, laser, laser tissue-interaction, near-infrared laser pulse, ophthalmology, retina, spot size variation, wound healing, ultrashort laser pulse.

1. INTRODUCTION

Previous studies have reported the retinal damage thresholds stemming from single ultrashort laser pulses from visible wavelengths and from single laser pulses from near infrared wavelengths[2]. In this study we analyzed the retinal damage thresholds for large spot laser lesions from near infrared wavelengths and are comparing them to the single small spot laser lesions from near infrared and visible wavelengths reported previously.[2-13]

2. PURPOSE

We wish to identify whether there is a greater retinal tissue injury due to varying spot size of the laser pulse by comparing large spot size near infrared (1060 nm) laser pulses to the retinal effects from small spot size near infrared (1060 and 1064 nm wavelength) laser pulses and small spot size ultrashort (532 and 580 nm wavelength) picosecond and femtosecond laser pulses.

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3. METHODS

3.1. Experimental Systems

The laser system used to produce 804 micron large spot laser lesions operated with a mode-locked Ti:Sapphire oscillator operating at 76 MHz, 1060 nm, and a pulsewidth of 150 fs. Single pulses at 150 fs with energies up to 3 mJ were provided by a doubled Nd:YAG-pumped Ti:Sapphire Regenerative Amplifier. Measurements of pulsewidths were always taken with a slow scan autocorrelator with the compressor set for minimum pulsewidth. A shuttered, Krypton laser beam, adjusted to give a 3 ms at 3 watts output power, was used to attain marker lesions.

Placement of the beamsplitter approximately 1 cm from the cornea allowed the reflected beam to enter the eye. The energy of each pulse was measured by directing the transmitted portion of the beam into an energy meter. The actual energy delivered to the cornea was calculated for each pulse by using the measured ratios of the reflected and transmitted portions of the beam. Measurement of these energies and ratios was performed with a Molectron JD2000 joulemeter/ratiomenter with J4-09 or J3-09 detectors.

3.2. In Vivo Model

Standard laboratory conditions (12 hours light, 12 hours dark) were used to maintain 2.2 to 6.9 kilogram (kg) mature macaca mulatta primates. Pre-exposure screening was performed on all primates to verify that no eye was more that one-half diopter from being emmetropic. All procedures were performed during the 12 hour light cycle.[2]

3.3. In Vivo Preparation

All animals were sedated using intramuscular injections of 10 milligrams (mg)/kg ketamine hydrochloride (HCl). Once sedated a subcutaneous injection of 0.16 mg atropine sulfate was administered. Each eye was treated with proparacaine HCl 0.5%, phenylephrine HCl 2.5%, and 1% tropicamide. Propofol was administered through an intravenous catheter. Anesthesia was maintained with 0.2 - 0.5 mg/kg/min doses of propofol via syringe pump with monitoring of temperature and vital signs. Extraocular muscular movement was reduced by administration of a peribulbar injection of 2% lidocaine. Fundus photography, laser exposure, and fluorescein angiography (FA) were performed. For fluorescein angiography, 0.6 ml of fluorescite 10% (Alcon Laboratories) was administered intravenously via rapid bolus.

Saline solution (0.9%) was used to keep the cornea moistened throughout our procedures. A modified fundus camera was used to view the retina at all times and exposures (9 to 16) were delivered to the macula in a rectangular grid pattern. Eyes used for the large spot size lesions were fitted with a flat contact lens to eliminate the curvature of the cornea. A +4.5 D lens was placed at a distance of 9 cm from the cornea to give a retinal spot size of 804 microns. For longer pulse widths, creation of visible retinal marker lesions was achieved by shuttered exposures of the mode-locked, doubled, compressed Nd:YAG output at 82 MHz. For the 1 ps and 150 fs pulse exposures, a 3 millisecond shuttered exposure of Krypton laser output was used to create visible retinal marker lesions. Exposure sites were applied within a L-shaped grid pattern of columns and rows. Researchers observed lesion formation and captured fundus and fluorescein photos through the fundus camera's optical system. For FA, Black and white fundus photographs were taken directly before dye injection, during fluorescein angiography, and then every few seconds until a 5 minute time span was completed. This provided sequenced photographs showing the development of fluorescein leakage which was later graded from the photographs.

All eyes were evaluated at 1-hr and 24-hr post-exposure by at least two examiners. Lesions at a given exposure site were only reported as visible lesions if both examiners identified a lesion. Black and white photographs of the FA and color fundus photographs were taken at 1-hr and 24-hr post-exposure.

3.4. Statistical Analysis

The Probit Procedure[14] was utilized to approximate the ED_{50} dose for producing a minimal visual lesion (MVL) in the retina for all pulsewidths and to gauge the 95% confidence intervals for the ED_{50}s. Sufficient data was collected to guarantee that the fiducial limits remained within predetermined boundaries for visible lesions at 24 hours post-exposure. The upper fiducial limit could not be larger than 50% greater than the ED_{50} dosage and the lower fiducial limit could not be any less than 50% of the ED_{50} dosage.[15]

3.5. Tissue Preparation/Examination

Following enucleation the globes were incised and immersed in 3% Glutaraldehyde and 0.1M sodium cacodylate buffer. After 10 minutes the posterior eye cup was separated from the anterior segment and placed back into the fixative. The macula was dissected and embedded in Spur's resin. The embedded tissue was then sectioned in 1μm sections and stained with toluidine blue for light microscopy. Images were captured digitally using Scion Image® and readied for publication with Adobe Photoshop®. The degree of retinal damage was compared to our previously published data by using a modified version of our previous grading scale.[1]

- The animals involved in this study were procured, maintained, and used in accordance with the Federal Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources, National Research Council.[2]

4. RESULTS

The 150 fs near infrared wavelength large spot laser lesions were remarkable in their clinical and pathological appearance. The lesions, rather than centering on a single focal lesion as typically seen in pulsed laser lesions of the retina, demonstrated a spotted pattern of multiple focal lesions across the area of laser delivery.

Fundus photographs demonstrate the punctate pattern of fine white lesions approximately 10 to 15 microns in diameter scattered across the 800 micron site of laser delivery with normal appearing spaces between the punctate pale lesions. There was no site of more intense central damage in the lesion, and there was no peripheral ring of pigmentary change. There were no hemorrhages with energy delivery as high as seven times threshold. Lesions created by energy of 0.7 to 1.1 times threshold covered a smaller area than those created by 3 to 7.9 times threshold. The calculated ED_{50}[16] for a minimal visible lesion at 24 hours was 54.1 μJ. The minimal energy that produced a visible lesion was 31μJ.

Histopathologic examination of 24 hour old lesions revealed that the 150 fs, 1060 nm large spot size laser lesions had minimal to moderate damage in the retinal pigment epithelium. The minimal energy that produced a visible pathologic change was 37 μJ. The RPE response was notably mild in the low energy lesions, with multiple sites of focal injury to one or two cells, with rare large vacuoles or migration of RPE cells. In higher energy lesions there was a more confluent area of RPE disturbance including stacking of RPE cells as can be seen at this duration after injury.

There was focal photoreceptor injury in very few lesions of energy 2 or more times the ED_{50} threshold. Despite the broad size of the beam, the focal photoreceptor injury extended across a 20 to 30 micron site. Multiple sites of focal photoreceptor injury could be seen in a single lesion. Rarely the outer retinal injury extended into the outer plexiform layer. Inner retinal layers appeared normal. Bruch's Membrane remained intact in all lesions. There were several focal sites of possible mild choriocapillaris damage in higher energy lesions.

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3. Int Journal of Cancer, 31 May 1999, 81(5):808-812
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Microwaves, Magnetic Iron Particles and Lasers as a Combined Test Model for Investigation of Hyperthermia Treatment of Cancer

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There is renewed interest in the use of hyperthermia, both generalized and localized, for the treatment of cancer. Proof of the effect of hyperthermia in cancer has been shown by Block [9] with the effect of heat on tissue cultures of LZ 100 leukemia cells. Other investigators [17] have also shown that for tissue cultures hyperthermia alone can kill tumor cells at levels usually 41–43° C. Overgard and Overgard show selective effects of 41.5–43.5° C on mouse mammary tumor implants without any effects on adjacent normal tissues. Hyperthermia has been used extensively in clinical studies with warm packs, blankets, infrared cabinets, paraffin baths, and even for limb perfusion experiments with heated blood and chemotherapeutic agents [2].

Various forms of radiation techniques also have produced tissue hyperthermia. These include microwaves [10], radio frequency waves [4, 5] cauteries [1] including infrared coagulator and lasers [6, 8]. Microwaves are a form of electromagnetic energy with a frequency range of 10^9–10^{11} MHz and a wave length from 30 cm to less than 0.3 cm. Most medical microwaves for tissue heating are 2450 MHz. Microwaves have poor penetration in muscle and fat tissue. Guy et al. [3] believe that 915 MHz should be used for deeper penetration. As yet, this is not available. Microwaves stimulate cellular metabolism even at energy densities of 5 MW/cm^2. Measurement of actual output of microwave in tissue is still difficult [12]. The National Cancer Institute [11], at present, is interested in microwaves in the treatment of cancer, even for non-thermal effect.

Recently, induction heating has been done by radio frequency type (RF) [4]. A radio frequency generator output is coupled to the two sharp electrodes closely spaced to one another. The energy confined to the area between the two electrodes does the cutting, the so-called radio frequency knife. With radio frequency energy (RF), LeVeen and his associates [5] have shown animal and human cancer tempera-

ture rises to 5–9° C without destruction of normal tissue. In their series, RF energy produced "necrosis or substantial regression of cancer in 21 patients." This type of energy may be superior to microwaves regards deeper penetration in tissue.

To increase the tissue hyperthermia induced by microwaves in rabbits, in our experiments various iron compounds were deposited in tissue. With some of these iron compounds, also strong magnetic fields may be used to move the particles through tissue. This adds the biological effects of magnetology as another adjuvant. Suspensions of one of these compounds, ferrofluids, can be moved by magnetic fluids and is non-toxic to local tissues. The dark color of the iron compounds also increases absorption by lasers in the visible and near infrared light range [8]. Those iron particles, moved by magnetic fields, can be mobilized and fixed deeper in tissue. The laser beams can impact only accessible or superficial tissues unless transmitted by special wave guides deep into tissue [8]. All these various modalities together then can make for selective local hyperthermia even to metastases. The lasers used in our experiments include:

1. ruby laser 694.3 nm: energy densities 75–90 J/cm^2; 2.5 ms exposures; target area 2.1 cm^2.
2. argon laser 488.8–5145 nm: 3 watts; 1–2 s.
3. ND-YAG: 1060.0 nm; 30 watts; 1–2 s.

In the microwave and laser experiments, 8 rabbits were used as the experimental animals with an average weight of 2 kg. The anesthesia was Ketaset 33 mg/kg, i.m. Small pockets were made in the skin by small parallel incisions in the epilated abdominal skin of the anesthetized rabbits. The iron materials were injected into these pockets. To increase microwaves absorption, various forms of iron particles were used. These include:

1. micro crystalline iron particles: size 6–9 μ.
2. ferrofluids: micro-encapsulated colloidal particles 100 A in size (Ferrofluidics Corporation–Burlington Mass., U.S.A.). The saturation magnetization here is considered to be 300 gauss with viscosity of 10 centipoise at 30° C. Vehicles used included water and fluorocarbons.
3. Imferon B: iron dextran complex formed by ferric dioxide and dextran. This material is given usually by intra-muscular injection for the treatment of anemia.

All the iron compounds produced intense coloration of tissue. This made for increased absorption of the laser system used. To study changes produced in the

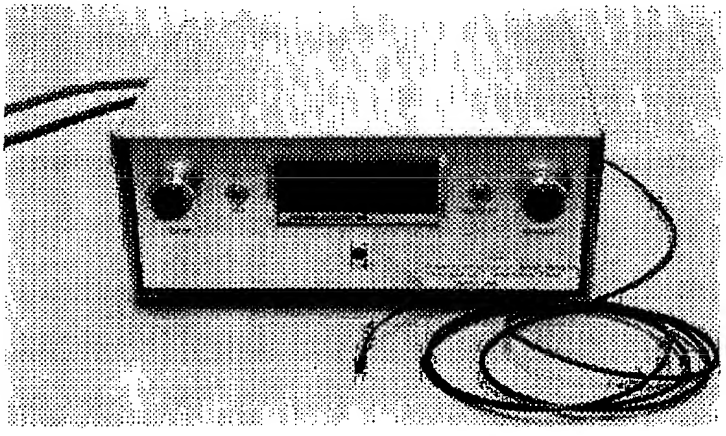

Fig. 1
Rozzell Liquid Crystal Optical Probe (LCOF) a non-metallic temperature sensor for tissue temperatures

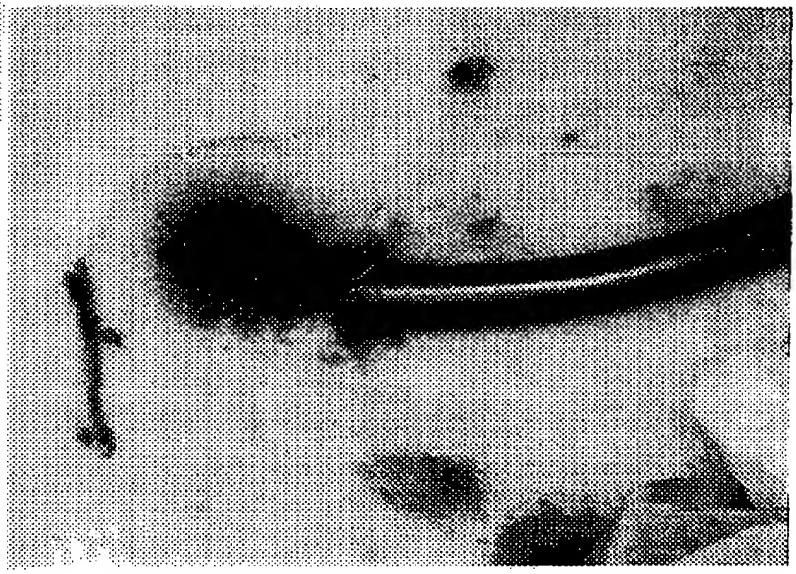

Fig. 2. LCOF Probe, a superficial skin pocket in the epilated abdominal skin of the rabbit after insertion of ferrofluids

iron compounds by laser beams, suspension or smears of the iron compound were put on glass slides, impacted by laser beams, and the impact areas observed under the microscope. Iron particles and ferrofluids showed clear-cut zones in the laser impact areas. The Imferon showed crystalline iron particles about the periphery of the clear impact zone. The magnetic fields moved the fresh iron particles and ferrofluids on the glass slides and about the surface on gelatin block phantoms. The Imferon was not moved by magnetic fields.

After injection of the various iron compounds into the skin pockets of rabbits, the surface was then cleansed. Following this, the temperature sensors were inserted. The LCOF (liquid crystal optical fiber) sensors were the only ones used during actual exposures to microwaves. The other temperature sensors, standardized thermometers, were used immediately before and after exposures to microwaves and the lasers.

To measure tissue temperature induced by microwaves and during exposure to microwaves, we have used the Rozzel liquid crystal probe for measuring tissue temperatures. Metal clad thermistors or thermocouples cannot be used. The liquid crystal optic fiber (LCOF) temperature probe utilizes solid state opto-electronic components to convert the transduction phemomenon occurring at the liquid crystal fiber into a digital temperature results. Temperature readings were taken from the normal skin, skin over the pocket, and from inside the pocket containing the iron compounds. In the laser experiments, impacts were delivered into the open pocket area.

Biopsies were taken from the iron depot areas and adjacent normal skin. The animals were sacrificed after the microwave and laser impacts. No body temperature readings were taken of the animals. The biopsies did show diffuse spread of iron particles with globular masses, intracellular iron particles observed with iron stains. There was some lymphocytic infiltrate and an intense histiocytic response.

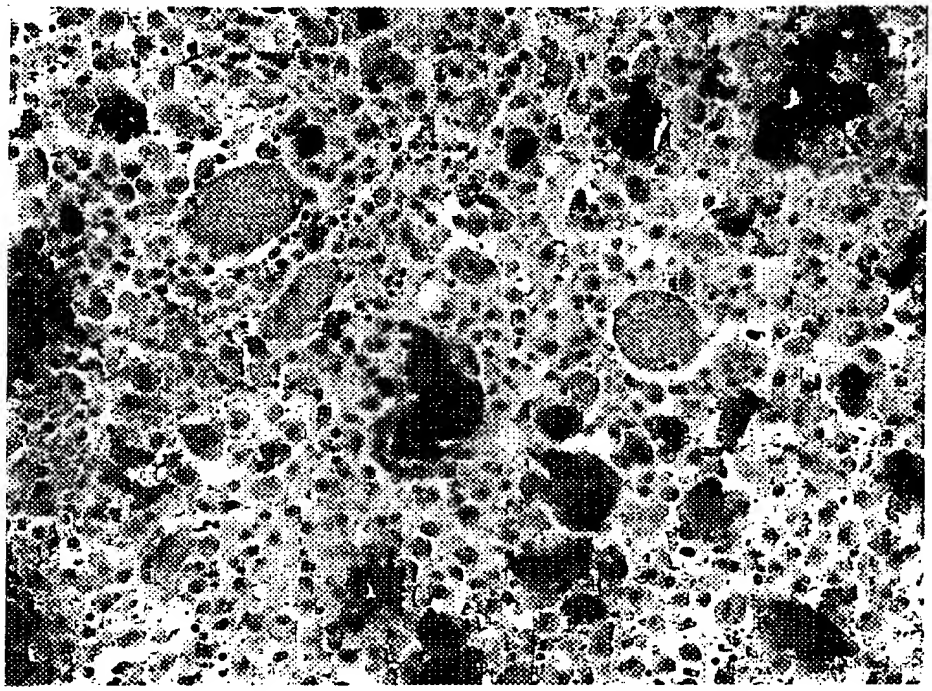

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Fig. 3. Section showing globular iron masses diffuse histiocytic response with intracellular ferrofluid particles after microwave irradiation hematoxylin-eosin × 200
of impact area

Fig. 4. Ruby laser impact, 75 joules/cm^2, 2.5 ms, 2.1 cm^2, on smear of Imferon on slide showing complete vaporization of the material with scattered micronized metallic iron particles about the periphery

Table 1. Average temperature measurements of skin pockets of 8 rabbits

	(LCOF Probe)	
	before microwave (°C)	after microwave (30 min) (°C)
No iron particles	34.2	39.0
Ferrofluids (fluorocarbon)	34.2	49.0
Ferrofluids (aqueous)	35.0	54.5
Imferon	34.2	51.5
Micro crystalline iron particles		
25 cc suspension	34.2	43.5
50 cc suspension	34.2	45.0

Table 2. Average comparative temperatures–Micro crystalline iron particles (Microwave 100%; Power 4"; 30 min; 2450 MHz)

Control	LCOF (°C)	Standard thermometer (°C)
No iron	39.2	39.0
Micro crystalline powder (25 cc aqueous suspension)	42.0	41.5
Micro crystalline powder (50 cc suspension)	45.0	44.0

(LCOF Probe used during exposure B. standard thermometers used immediately after microwave exposure)

No thermal coagulation necrosis was observed except after laser impacts. Control pretreatment biopsies showed less histiocytic response. In postmortem examinations, when the animals were sacrificed, pulmonary edema was found in several of the animals. With laser impacts, deep necrosis was produced with all lasers.

Controls included:

1. tissue pockets alone
2. microwave exposure
3. laser impact exposure
4. iron compounds without exposure

It is evident that with this test model, with exposure to microwaves, a superficial depot of various types of iron particles produced local tissue hyperthermia of a significant intensity (Table 1). The temperature differences between the various iron compounds are not meaningful in these few experiments, although the micro crystalline iron deposits did give lower levels (Table 2). More controls would be necessary here as regards numerous factors such as homogeneity of masses and distribution particles, type and location of temperature sensor, microwave measurements in selected areas of the pocket, controls with other techniques for the induction of hyperthermia, etc.

No direct temperature measurements were done during or after laser impacts. In previous experiments with laser surgery of the liver in dogs, tissue temperature

measurements, by thermistors, after laser impacts showed little heat transmission to adjacent tissues. This was from the CO_2 laser [8].

A patient with basal cell carcinoma was treated by low energy density ruby laser after local infiltration of the basal carcinoma with ferrofluids. The patient has now been observed for over 3 years without any recurrences of the carcinoma or any toxic changes in the skin [13].

Therefore, with the current interest in hyperthermia treatment of cancer [14, 15], it is necessary to do controlled investigative studies on microwave and radio frequency technology (RF). One can repeat the conclusions of Block and Zubrod [16] that, "well designed clinical efforts to unequivocally evaluate the effects of hyperthermia on cancer are critically needed". This lack of controls in experiments on tissue hyperthermia is also emphasized by Bull and Chretien [17] of the National Cancer Institute, "Rationally designed and properly controlled studies are now warranted by the present background of data derived from laboratory studies providing a theoretical basis for hyperthermia therapy and by clinical reports that show feasibility and efficacy of heat therapy". The dermatologists should accept the challenge.

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Effects of transpupillary th rmoth rapy on immunological paramet rs and apoptosis in a case of primary uveal melanoma

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The Department of Ophthalmology, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands. Tel: (+31) 71 5263938; Fax: (+31) 71 5248222; Email: schurmans@worldonline.nl (L. R. H. M. Schurmans, D.-J. R. Blom, I. De Waard-Siebinga, J. E. E. Keunen, M. J. Jager). Eye Pathology Institute, University of Copenhagen, Copenhagen, Denmark (J. U. Prause).

Transpupillary thermotherapy (TTT) is a new treatment modality for uveal melanoma. We studied whether application of TTT influences the immunogenicity of the tumour cells *in vivo* or the expression of molecules related to apoptosis. Immunohistochemistry using monoclonal antibodies directed against HLA molecules, HMB45, P53, Fas ligand (FasL), Fas, Bcl-2 and tumour-infiltrating cells was applied to sections of an enucleated eye containing a uveal melanoma that received TTT 1 week before enucleation. The innermost part of the tumour which had been exposed directly to the laser treatment showed no staining for HLA antigens, nor for Fas or FasL epitopes. The intermediate part of the tumour showed a wet necrosis and HLA expression similar to the expression in the peripheral tumour. A large number of macrophages were observed in the necrotic as well as the intact tumour tissue, especially bordering the wet necrotic area. FasL and Bcl-2 were only expressed in the viable, outer part of the tumour. This immunological evaluation of one case of uveal melanoma treated with TTT revealed that TTT may not only have a direct destructive effect on the primary tumour, but may also influence the immunogenicity of uveal melanoma cells, induce infiltration of macrophages into the tumour, and induce apoptosis. The presence of many macrophages suggests that they play a role in the removal of the TTT-treated tumour tissue by phagocytosis. © 1999 Lippincott Williams & Wilkins

Key words: apoptosis, HLA molecules, transpupillary thermotherapy, uveal melanoma

Introduction

Transpupillary thermotherapy (TTT) is used for the local treatment of uveal melanoma.[1] The first results of this experimental technique are promising, and TTT applied by diode infrared laser is now being employed clinically in several centres.[2] TTT is a form of long-duration low-intensity laser therapy, and offers excellent heat penetration into the tumour. When TTT induces enough heat, tumour necrosis occurs. Histopathology reveals tumour cells with shrunken nuclei without nucleoli and cytoplasm (as observed in the study of Journée-de Korver *et al.*[3]), indicating tumour cell necrosis in the treated area.

We recently studied the *in vitro* effect of hyperthermia on HLA class I expression on uveal melanoma cell lines, and observed that *in vitro* application of hyperthermia (41 to 45°C) resulted in decreased expression of HLA class I.[4] Previously, Davies *et al.*[5] observed a similar decrease in HLA class I expression on cultured human melanoma cells. This decrease was followed by a transient increase in expression above the level seen in untreated cells.

HLA antigens play an important role in the presentation of tumour-specific antigens to T-cells and natural killer (NK) cells.[6–9] Therefore, *in vivo* alterations in HLA expression by TTT could influence the immunogenicity of uveal melanoma cells, and change their capacity to present tumour-specific antigens to T-cells. Another important factor that may affect antitumour responses is the induction of apoptosis. Contact between tumour cells positive for Fas ligand (FasL) and T-cells expressing Fas will lead to killing of such T-cells, preventing the lysis of the tumour cell. To our knowledge, the present study is the first study in which a uveal melanoma treated with TTT and then enucleated has been studied by immunohistology. We determined whether TTT

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changes the expression of HLA class I molecules *in vivo*, induces apoptosis, or influences the presence of tumour-infiltrating immune cells.

Materials and methods

Patient and tumour

A 44 year old, previously healthy female presented with decreased vision in the right eye of 2 months' duration. The visual acuity was 20/25 in the right eye (OD) and 25/25 in the left eye (OS). Slit-lamp examination revealed no anterior segment abnormalities. Fundus examination revealed an elevated pigmented mass in the posterior pole of OD, touching the optic disc along the temporal-inferior border. On ultrasonography, the basal diameter of the lesion was 9.8 by 12.5 mm, with a maximum height of 4.5 mm. B-scan ultrasonography revealed a choroidal excavation and the A-scan showed a low tumour reflectivity. The tumour was clinically diagnosed as a juxtapapillary submacular choroidal melanoma. A metastatic work-up yielded no signs of metastasis. When enucleation was suggested, the patient suggested that the eye be used to support research into the use of TTT. Taking into consideration the psychologically heavy burden of intraocular melanoma and, moreover, the pain that is sometimes involved in TTT treatment, the uniqueness of the present material is clear. Non-curative TTT treatment[1] was performed after obtaining fully informed consent from the patient, followed by enucleation 1 week later. The study protocol had been approved by the Ethical Committee of the Leiden University Medical Center. The study agreed with the principles of the Universal Declaration of Human Rights and the European Convention for the Protection of Human Rights and Fundamental Freedoms.

At surgery, an artificial orbital prosthesis was placed. Histopathological examination by an ocular pathologist, Dr D. de Wolff-Rouendaal, revealed a mixed-cell choroidal melanoma.[3] The number of tumour vessels was low and no extrascleral outgrowth or optic nerve ingrowth could be detected. Mitoses occurred only sporadically.

HLA typing

HLA typing on peripheral blood lymphocytes by standard complement-dependent antibody-mediated cytotoxicity testing revealed that the patient had the following HLA type: HLA class I – A2, A3, B5, B35, B51, Bw4, Bw6, Cw4; HLA class II – DQ3, DQ4, DQ8, DR4, DR8, DR53.

Immunohistochemistry

Immediately after enucleation, part of the tumour was removed, snap-frozen in liquid isopentane and stored at −80°C until sectioning for immunohistochemical staining. The remainder of the specimen was processed for standard histopathological examination in Leiden and Copenhagen.

Detection of tumour-infiltrating cells and HLA class I antigens

The following anti-HLA class I monoclonal antibodies were used for immunohistochemical staining: W6/32, against HLA class I;[10] BBM1, against β2-microglobulin;[11] MA2.1, against HLA-A2;[12] and GAP-A3, against HLA-A3.[13] The following monoclonal antibodies were used for infiltrate analysis: Leu-4, specific for CD3 (Becton Dickinson, San Jose, California, USA); RIV6, specific for CD4 (Becton Dickinson); FK18, specific for CD8 (Dako, Glostrup, Denmark); Leu 19, specific for CD56 14; and Dako macrophage, specific for CD68 (Dako). A three-step immunoperoxidase technique was employed as described previously.[15] Negative controls were performed with phosphate-buffered saline/bovine serum albumin 1% (PBS/BSA 1%) replacing the primary antibodies. The slides were examined independently by two observers and interobserver disagreements were solved by studying sections together.

Detection of Fas and FasL

Deparaffinized sections were immunostained for the presence of FasL using the ChemMate Detection Kit (LSAB, Dako) and the following antibodies against FasL: C-20 diluted 1:10, 1:50 and 1:100 (sc-957, Santa Cruz Biotechnology), Q-20 diluted 1:10, 1:50 and 1:100 (sc-956, Santa Cruz Biotechnology), anti-FasL diluted 1:5, 1:20 and 1:50 (F37720, Transduction Laboratories, Lexington, USA), APO-1 FasL diluted 1:5, 1:20 and 1:50 (804-009-C100, Alexis Corporation, Läufelingen, Switzerland), and NOK-1 diluted 1:5, 1:20 and 1:50 (65330C, PharMingen, Hamburg, Germany).

Using the same technique the sections were

examined for the presence of Fas epitopes using the following antibodies against Fas (CD95): anti-APO-1 diluted 1:10 and 1:5 (anti-CD95, clone APO-1, Dako) and anti-FAS diluted 1:25 and 1:10 (anti-CD95, clone DX2, Dako). Expression of the Bcl-2 epitope was investigated using the LSAB technique with anti-human Bcl-2 oncoprotein diluted 1:60 (clone 124, Dako).

Deparaffinized sections were heated in a microwave oven for 2 × 5 min in retrieval buffer (S2031, Dako) and left to rest in retrieval buffer for 20 min at room temperature. Sections were washed with PBS buffer pH 7.0 (Dako S2034) for 5 min and incubated with primary antibody diluted with 2% BSA (A-7906 Sigma) for 25 min. A second washing step was performed with PBS buffer pH 7.0 for 5 min, followed by incubation with secondary biotinylated antibody ('AB2', Dako K5003) for 25 min. The sections were washed again for 5 min, blocked for 8 min (using 'HP', Dako K5003), and washed with PBS buffer pH 7.0 for 5 min. After incubation with streptavidin peroxidase ('HRP', Dako K5003), the slides were washed for 5 min and stained with AEC ('Chrom', Dako K5003) for 15 min. A final washing step was performed in tap water and the slides were stained with Mayer's acidic haemalun and finally fixed with Aquamount (Gurr, BDH Laboratory Supplies, Poole, UK). Control sections were run in parallel, omitting only the primary antibody.

Results

Histology

Macroscopic examination showed a choroidal melanoma with 4 mm depth of necrosis in the TTT-treated part. Between the necrotic and the apparently viable part of the melanoma a sharp demarcation was observed. Bruch's membrane and the pigment epithelium were mainly intact, with a partial interruption on the top of the tumour. In this area, a multilayered serous membrane was seen below a partly detached, necrotic retina.

The tumour was composed of three distinct areas (Figure 1). In the peripheral part, close to the sclera,

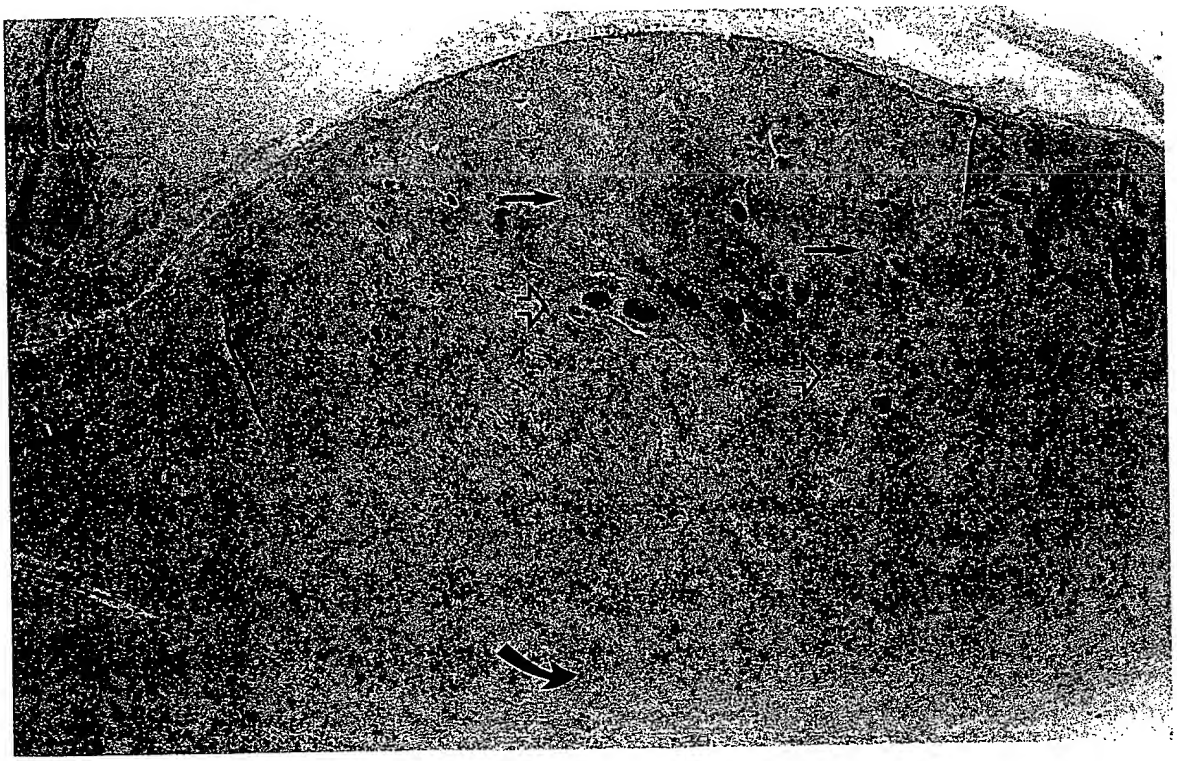

Figure 1. Survey of the TTT-treated tumour. Filled arrows mark the border between the innermost dry necrosis and the wet necrosis zone. Open arrows mark the border between the wet necrosis and the outermost viable tumour part. The curved arrow marks the internal scleral level (haematoxylin and eosin staining, original magnification × 30).

an apparently viable choroidal melanoma was observed, composed of a mixture of spindle cells and epithelioid cells with a number of vessels containing tumour cells. Only a slight inflammatory reaction was present in this area, and included pigment-loaded macrophages. In the middle of the tumour, a band of massive necrosis and haemorrhage was seen. In this area, many vessels were observed, with massive bleeding (wet necrosis). In the inner part of the tumour (the area facing the TTT beam) a dry necrotic zone was observed, with one small island of small tumour cells. These cells possessed pyknotic nuclei and might have been inactive; under the light microscope it was not possible to judge if these cells were viable.

Immunohistochemistry

All the antibodies reacted with their controls. The peripheral tumour part, containing apparently viable choroidal melanoma, showed proper expression of backbone HLA molecules and β2-microglobulin, and intermediate staining for HLA-A2 (Table 1). In the middle part of the tumour, increased staining for HLA-A2 was observed, while expression of HLA class I and β2-microglobulin were similar to that observed in the peripheral tumour part. The dry necrosis area showed no reactivity with any of the anti-HLA or β2-microglobulin monoclonal antibodies tested. FasL was demonstrated in the viable outer part of the tumour (Figure 2). The wet and dry necrotic parts did not show any staining. The same pattern was found for Bcl-2 reactivity. Fas epitopes were sparse. Fas was expressed on a few melanophages in the viable segment of the tumour and in the macrophages in the two necrotic layers of the tumour. However, neither the viable cells of the tumour nor the necrotic areas of the tumour showed any binding of the anti-Fas antibodies.

Staining for S-100 showed proper reactivity with the nerve fibres and a marked difference in staining of the three areas. While tumour cells in the peripheral part of the tumour were positive for S-100, the wet necrotic zone was completely negative. A positive reaction with anti-S-100 was present in the dry necrotic innermost part of the tumour, where especially the nuclei were stained. Staining for PCNA showed scattered positive nuclei mostly in the peripheral viable zone; only very few cells were stained in the dry necrotic zone and none in the wet necrotic zone. Staining for P53 was negative in all areas.

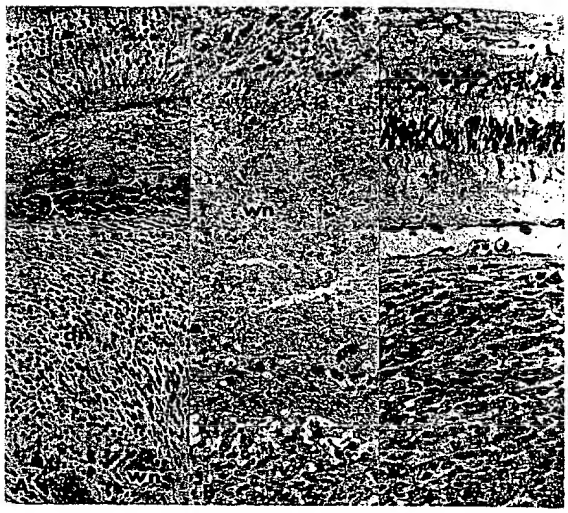

Figure 2. A Central area of the choroidal tumour; tumour cells do not react with antibodies to FasL. The cytoplasm is empty and the nuclei small and pyknotic, indicating dry necrosis (dn). At the bottom of the picture the transition to wet necrosis (wn) is noted. **B** Deep part of the central tumour. The transition between wet necrosis (wn) and viable tumour cells (v) can be seen. Note that the tumour cells in the wet necrosis do not express FasL, while the viable tumour cells do. **C** Peripheral, untreated part of the tumour, with viable (v) and markedly FasL-reactive tumour cells.

Immunocytochemistry

The only tumour-infiltrating immune cells observed were CD68+ macrophages. No tumour-infiltrating CD3+/−, CD4+/− or CD8+/− cells were observed. Some macrophages were observed in the dry necrotic area, while pigment-loaded CD68+ macrophages were especially seen in the middle part of the tumour, separating the innermost and peripheral parts of the tumour.

Discussion

We studied the effect of TTT on the expression of HLA molecules, Fas and FasL in a uveal melanoma that had received an experimental, non-curative TTT treatment 1 week before enucleation. Using cultured uveal melanoma cells, we have previously found that application of hyperthermia or X-ray irradiation results in decreased expression of HLA class I.[4] Whereas tumour cell recognition by cytotoxic T-cells needs expression of HLA class I molecules, the presence of these molecules may turn off NK cells.[16,17] In a retrospective analysis of 30 patients with uveal melanoma we recently demonstrated that

Table 1. Expression of different antigens in the three parts of the uveal melanoma after TTT was applied to the innermost part

	Peripheral part (apparently viable)	Transition part (wet necrosis)	Innermost part (dry necrosis)
HLA class I	++	++	–
β2-microglobulin	++	++	–
HLA-A2	+	++	–
HLA-A3	++	++	–
Bcl-2	+[a]	–	–
FasL	+	–	–
Fas	–[b]	–	–
HMB45	+	–	+[c]
P53	–	–	–
S-100	+	–	+[d]
PCNA	+	–	+/–[e]

[a]Marked cytoplasmic staining.
[b]A few positive melanophages, but no positive tumour cells.
[c]Staining of the cytoplasm was more pronounced than in the peripheral part.
[d]The nuclei were especially stained.
[e]Only a few positive cells.

high expression of HLA (-A as well as -B) is strongly correlated to shorter patient survival.[18] Our finding that tumours with a low expression of HLA class I usually did not induce metastases supports a role for NK cell mediated lysis of tumour micrometastases in the blood. We examined the possibility that application of TTT would change the HLA class I expression, and thereby the sensitivity of the uveal melanoma cells to NK cells. We found a dual effect with regard to HLA expression; on the one hand, a total loss of class I expression occurred in the dry necrotic part of the tumour, while on the other hand increased expression of HLA-A2 was observed in the intermediate zone. The immunological effects of this treatment may therefore be ambiguous (see below).

We observed a sharp demarcation between the necrotic and the apparently viable tumour parts. Such a demarcation zone following TTT has also been described by Journée-de Korver *et al.*[3] In the dry necrotic part of the tumour, some tumour cells were observed, but it was not possible to assess their viability by light microscopy. However, none of these potentially viable cells were found to express any HLA antigens.

In the middle part of the tumour we observed a markedly increased staining for HLA-A2 compared with the peripheral tumour part. Since large numbers of tumour-infiltrating macrophages were observed in this part of the tumour, immune responses mediated by macrophages could play a role in the removal of the tumour cells.[15,19] It may be that other types of immune responses, such as the immunological cytotoxic T-cell (CTL) and/or NK cell mediated antitumour responses, are inhibited intraocularly due to local circumstances.[20–22] In line with previous studies where only small numbers of infiltrating cells were found in uveal melanoma,[15,23,24] we did not observe any tumour-infiltrating CD3+/–, CD4+/– or CD8+/– cells inside the tumour. Macrophages may be responsible for the upregulation of HLA-A2 expression through local production of cytokines. Expression levels of HLA-A3 and monomorphic HLA class I antigens were high already, a possible local upregulating effect on these antigens could be masked. Although the peripheral part of the tumour appeared to be viable, apoptosis seemed to have been induced in this area. Many cell-specific mechanisms may initiate the apoptotic cascade.[25] One potent activator is the FasL-Fas system.[26] FasL and Fas are both members of the neural growth factor/tumour necrosis family. Cells expressing Fas on their surface, like cytotoxic T-cells, are pushed to apoptosis upon contact with cells expressing FasL. The FasL-Fas reaction then leads to cell death, which may be inhibited by interaction with Bcl-2.[25] Expression of Bcl-2 is common in tumour cells. The present finding of FasL expression in malignant melanoma is new. FasL expression may be responsible for the strikingly low number of lymphocytes found in malignant choroidal melanomas in contrast to cutaneous melanoma. The tumour cells might protect themselves from activated T-cells via FasL expression, a mechanism that has recently been suggested to be an important factor in the immune privilege of the eye.[26–28] The retinal pigment epithelium expresses FasL and is able to induce apoptosis in activated T-cells *in vitro*.[28] While neither the dry nor the wet necrotic layers of the tumour expressed FasL or Bcl-2, these layers harboured an increased number of Fas-positive lymphocytes and macrophages. One of the authors (J.U.P) has now seen this phenomenon in four other TTT-treated uveal melanomas (unpublished observations).

Application of TTT in combination with ruthenium plaque radiotherapy leads to regression of the melanoma in most cases, without a noticeable intraocular inflammatory response.[1] It seems likely that, following TTT, the necrotic part of the tumour is removed by macrophages.

In summary, we observed that TTT decreases HLA expression, while adjacent (potentially viable) tumour tissue showed an increase in expression of HLA-A2 and an increased presence of macrophages. Together with the loss of Bcl-2 protection against apoptosis, these observations illustrate different mechanisms by which tumour cells are removed from the eye.

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Treatment of Hepatocellular Carcinoma

JORDI BRUIX

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide.[1] In most of the cases, HCC develops in the setting of cirrhosis. Thus, cirrhotic patients constitute the population at risk, their 5-year probability of developing an HCC being around 20%.[1-4] This has prompted the screening of cirrhotic patients for HCC, which is usually performed by regular ultrasound examination and α-fetoprotein determination. This policy has facilitated the detection of HCC at an early and/or asymptomatic phase when potentially effective treatments are available. However, the lack of controlled trials for most therapeutic options precludes knowing if their antitumoral effect is associated with an improved survival. Frequently, the reported benefit is estimated by comparison with the outcome of historical cases thought to share the same baseline status. However, it must be stressed that the prognosis of HCC patients is determined not only by the HCC stage, but also by the functional status of the underlying liver.[5] Thus, prospective trials or retrospective analysis should be designed to avoid a bias caused by an incomplete baseline evaluation, which should include prognostic variables related both to the underlying cirrhosis (ascites, serum sodium concentration, renal failure, prothrombin time, bilirubin, albumin) and to the HCC stage (number and size of the nodules, vascular invasion, extrahepatic spread, presence of symptoms).

This review summarizes the efficacy of the most common therapeutic options for HCC, taking into account not only their antitumoral efficacy, but also their consequences on survival.

SURGICAL RESECTION

Resection is usually restricted to patients with solitary HCC without vascular invasion or extrahepatic spread. Extensive lobectomies are contraindicated in cirrhotic patients, and segmentary or subsegmentary resection should be the goal.[6] A tumor diameter $\leq$5 cm was frequently used as a cutoff because larger tumors were more likely to present additional nodules, and thus, incomplete resection or early disease recurrence were common events. However, with newer imaging techniques (spiral computed tomography, magnetic resonance), the additional tumor nests are more easily identified preoperatively, and thus, a large HCC classified as solitary may indeed be solitary, and may still be suitable for successful surgery despite its size. Liver function should be preserved, and this cannot be assessed merely by the preoperative Child-Pugh classification. Despite selecting only Child's A patients for surgery, more than half of them will develop hepatic decompensation (namely ascites) after resection, and if this does not resolve within 3 months, it constitutes an indicator of poor prognosis.[7] We have recently shown that the presence of significant portal hypertension (hepatic venous pressure gradient $\geq$10 mm Hg) is the best predictor of unresolved postoperative hepatic decompensation, being more accurate than any other parameter including the indocyanine green metabolism.[7] Accordingly, surgical resection should be proposed only in patients with extremely well-preserved liver function (serum bilirubin should be normal because increased values are associated with decompensation and poorer outcome) and no portal hypertension. These patients will not present relevant functional impairment, and thus, their medium and long-term survival will not be impaired by surgery. Using these selection criteria, the 5-year survival exceeds 50%, there being no differences between Western and Japanese studies.[6-9] The main drawback of tumor resection is the high recurrence rate, which may exceed 50% after 5 years of follow-up.[6-9] New HCC foci may be the result of dissemination from the primary tumor or may be a metachronous HCC in a predisposed cirrhotic liver. Pathological data such as the absence of pseudocapsule, vascular invasion, or presence of additional nodules constitute markers of high risk of recurrence. It is expected that the molecular analysis of the tumors will help to further define this risk. Adjuvant treatment with chemotherapy or preoperative chemoembolization has not been shown to be beneficial in reducing recurrence or improving survival.[10]

Patients at high risk of recurrence by pathological examination of the resected liver may benefit from orthotopic liver transplantation (OLT) even without evidence of residual tumor, this proposal being based on the fact that patients with small HCC showing the same pathological features present a very low rate of recurrence after OLT. However, the results of this strategy have not yet been reported.

OLT

While surgical resection treats only the HCC, OLT would cure both the tumor and the underlying cirrhosis, because the unresected liver not only is the most frequent site of metastatic nodules, but also is a premalignant condition. The initial series of OLT for HCC included mainly patients with advanced HCC, and they described a high recurrence rate.[11] Interestingly, however, it was also noted that patients with incidental HCC (not detected until the pathological examination of the explanted liver) had a very low recurrence rate.[11] This prompted several groups to exclude

Abbreviations: HCC, hepatocellular carcinoma; OLT, orthotopic liver transplantation; PEI, percutaneous liver injection.

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all known HCC patients from the OLT programs or to restrict the indication to those subjects with HCC detected at an early stage, i.e., solitary HCC ≤5 cm. Subsequent studies have confirmed that the stage of the HCC is a crucial factor for the outcome of the patients, and thus, the key point in determining the guidelines for OLT.[12-14] It has thereby been shown that, if OLT is restricted to patients with solitary HCC ≤5 cm or to subjects with less than three tumor nodules (each ≤3 cm), the recurrence is negligible and the survival (75% at 4 years) is very similar to that of non-HCC patients.[13,14]

The shortage of organ donors means that a serious risk that HCC patients face while waiting for OLT is the growth of the tumor, to the point at which it could lead at least to a higher recurrence risk and possibly to OLT contraindication. The length of this wait varies among different programs, but it emphasizes the need for analyzing OLT according to intention to treat, an approach that is frequently missed and that might offer devastating figures if the waiting period exceeded 6 months. Some authors perform chemoembolization, aiming to necrose most of the HCC and thereby reduce or delay tumor growth and dissemination during the waiting period. However, this approach must be evaluated by prospective controlled trials, because the treatment itself may promote tumor dissemination,[15] and it has been shown that a restrictive selection can almost abolish HCC recurrence, even without adjuvant treatment.[13,14] Finally, the role of different posttransplantation immusuppresive strategies in facilitating the survival and promoting the growth of neoplastic cells released from the tumor is unknown.

The results of OLT for HCC should not only focus on tumor recurrence and medium-term survival. Most of the HCC patients are infected with the hepatitis B or hepatitis C virus, and infection of the new liver is the rule.[16] Hyperimmune gammaglobulin and/or new antiviral drugs have shown to be useful for B virus, but there is no effective prevention or treatment for C virus infection,[16] the most common etiologic agent for HCC in Western countries.[1] Thus, chronic liver disease may arise in the new organ and re-establish the oncogenic potential of the liver and/or evolve to end-stage liver disease. All these considerations have tempered the enthusiasm for OLT and justify that, while waiting for the results of studies comparing OLT versus resection, most groups still consider surgical resection as the first option to be evaluated.

PERCUTANEOUS ETHANOL INJECTION

Percutaneous ethanol injection (PEI) has gained a wide popularity because of its high antitumoral efficacy coupled with its relatively simple and inexpensive application. Absolute ethanol is injected through a fine needle into the tumor on separate days under continuous ultrasound control; the infiltration of the HCC induces its complete necrosis, which is confirmed by the absence of contrast uptake on dynamic computed tomography and/or angiography.[17,18] PEI is well tolerated. Intense pain caused by ethanol leakage into the peritoneal cavity can be prevented by the slow injection of the ethanol and avoiding quick needle removal; the most common complaints are moderate pain during the infiltration and slight fever caused by HCC necrosis.[17,18] Rare but more serious complications are vascular thrombosis caused by the passage of ethanol into the portal vein, self-limited hemoperitoneum, and tumor dissemination along the needle tract. PEI is highly effective in solitary tumors ≤3 cm, in which more than 90% undergo complete necrosis.[17,18] However, in larger tumors, the success rate is significantly less, and even if complete necrosis is accomplished, HCC recurrence is frequent early during follow-up. To increase the efficacy of PEI in larger tumors, some authors have evaluated the usefulness of injecting larger volumes (under general anesthesia) or of combining PEI with prior chemoembolization, but the survival benefits of these approaches are unknown. Another potential option is the injection of acetic acid[19] or hot saline.[20] These fluids diffuse more easily within the HCC, and, in addition to being not harmful if entering vessels or painful if leaking, they would require less volume to embed and effectively necrose the tumor. Finally, the intratumoral placement of microwave electrode through a large-caliber needle has also been reported to induce complete tumor necrosis.[21]

In small tumors, the recurrence rate and pattern are similar to those following resection.[22] The high antitumoral efficacy and the absence of related mortality produce a survival rate that is almost the same as that of surgical patients,[22] exceeded only by that of surgical patients with normal liver function and normal portal pressure. In these selected cases, surgery has the advantage of completely resecting the HCC with the surrounding margin that may contain microscopic tumor foci.

TUMOR EMBOLIZATION AND CHEMOEMBOLIZATION

These options require the catheterization of a peripheral artery with advancement of a catheter into the hepatic artery selectively feeding the HCC. Arterial blood flow is obstructed by the injection of gelfoam or the placement of metallic coils. If the portal blood flow is not preserved, arterial obstruction is contraindicated. Ischemic necrosis affects more than 80% of the HCC in the majority of the patients.[23,24] Viable tumor nests may be caused by their portal blood supply, and thus, would only be affected by portal vein embolization. Arterial obstruction is associated with the development of fever, moderate abdominal pain, and segmentary ileus. This postembolization syndrome is self-limited, and, thereafter, most of the patients recover their baseline status. Flow obstruction is usually combined with the injection of chemotherapeutic agents suspended in lipiodol (a contrast agent that is selectively retained within the tumor), but there is no evidence that this chemoembolization is more effective than embolization alone.[25] Furthermore, despite the fact that the survival of patients with extensive tumor necrosis is better than that of patients with poorer responses,[23,24] prospective trials have failed to show an improvement in survival.[26] In addition, the disruption of the tissue architecture induced by ischemic necrosis may facilitate the dissemination of neoplastic cells,[15] ultimately counteracting the initial therapeutic benefits, which are lost within the first year of follow-up.[23] Thus, present efforts are directed at increasing the initial therapeutic effect and maintaining it for longer periods.

CHEMOTHERAPY

The response rate and the potential effects of chemotherapy on survival have been disappointing. This has been related to the expression of the multidrug resistance gene potentially expressed through p53 mutations,[27] which, in the absence of aflatoxin exposure, are usually restricted to advanced HCC. Because most of the drugs have been tested in patients with advanced HCC, it is possible that their efficacy may have been underestimated. Thus, there could be some rationale for re-evaluating some of these agents in patients with less advanced disease.

The selective injection into the hepatic artery of chemo-

therapeutic agents, usually adriamycin, suspended or not in lipiodol, does not increase the antitumoral action, reduce the systemic toxicity, or improve survival.[28] This is because of the low hepatic extraction of most of the drugs and their nonmodification by the suspension in lipiodol. Preliminary results using new agents with different pharmacokinetics appear promising but require confirmation.

HORMONAL MANIPULATION

Antiandrogenic treatment has never been shown to be effective, and it may be associated with serious side effects.[29] The lack of efficacy may be because of the fact that this option has been evaluated in patients with advanced HCC, a stage at which androgen receptors are usually absent.[30]

The data regarding antiestrogenic treatment have been more controversial. Administration of tamoxifen, an estrogen receptor blocker, was suggested to improve the survival of patients with advanced HCC.[29,31,32] Nevertheless, the largest double-blind, placebo-controlled trial offered negative findings,[33] it being debated if different dosages and the presence of mutated receptors may explain the discrepancies.

RADIOTHERAPY

Selective radiotherapy can achieve extensive necrosis in small, solitary tumors, but still carries the risk of inducing actinic hepatitis or producing bowel injury.[34] It has been reported that proton irradiation would be highly effective while lacking severe side effects.[35] The intraarterial injection of lipiodol mixed with ^{131}I has offered encouraging preliminary results,[36] but it requires more intensive study.

IMMUNOTHERAPY

Results with immunomodulators have been negative. However, the administration of interferon has been reported to improve survival when compared against adriamycin or placebo[37]; thus, this option awaits further evaluation.

CONCLUSION

In summary, the treatment of patients with HCC remains a clinical challenge with several areas to be investigated through carefully designed prospective trials. Ideally, this clinical research will provide us with solid therapeutic options that unequivocally improve the survival of the patients, which ultimately also should be the end-point of early detection plans.

Acknowledgment: The author thanks Eric Webber and Jonathan Schwartz for their valuable comments.

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SURGERY TODAY

Review Article

Indication of Liver Transplantation for Hepatocellular Carcinoma in Japan*

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Abstract: Approximately 20,000 patients die of hepatocellular carcinoma (HCC) annually in Japan and most of them are hepatitis B virus (HBV) or hepatitis C virus (HCV) carriers. Recently, small HCC, less than 3 cm in diameter, have frequently been found by ultrasonography in the follow-up of patients with chronic liver diseases. Such cases are mainly treated by either surgical resection or percutaneous ethanol injection therapy (PEIT) with a satisfactory 5 year survival rate of 50%. In addition, the survival rate of advanced cases has gradually improved thanks to transcatheter arterial chemo-embolization combined with PEIT, radiation, hyperthermia, or immune therapy. On the other hand, our autopsy study has indicated a high frequency of extrahepatic metastasis in advanced cases. From these results, liver transplantation for HCC does not seem to be the treatment of first choice, at present, in Japan. In the future, the means to control the underlying infection of HBV or HCV as well as making an accurate imaging diagnosis for the detection of extrahepatic metastasis will become inevitably more important for successful liver transplantation in HCC.

Key Words: liver transplantation, hepatocellular carcinoma, treatments of hepatocellular carcinoma

Introduction

It is important to evaluate the present status of medical management including the diagnosis and treatment of hepatocellular carcinoma (HCC) in Japan when we consider the liver transplantation of HCC. In Japan, approximately 40,000 die of hepatic diseases annually and this hepatic death rate to the general population is five times higher than that compared with the United Kingdom and three times higher than that in the USA (Table 1). Though this extremely high incidence of hepatic death indicates the great number of patients who need liver transplantation in Japan, of those about 20,000 die annually of HCC in Japan (Fig. 1). However, there is an extremely small number of liver donors available even if liver transplantation is soon started in Japan. In addition, the results of liver transplantation on HCC carried out in foreign countries have proven to be less than satisfactory.[1] For these reasons it is possible that HCC would not be positively indicated for liver transplantation for some time in Japan.

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Characteristic Features of HCC and Liver Transplantation

HCC has characteristic features, which differ from other malignant tumors: 1) over 80% of HCC are associated with liver cirrhosis, 2) over 80% of HCC patients are hepatitis B virus (HBV) or hepatitis C virus (HCV) carriers, 3) multicentric carcinogenesis within the liver is often present, and 4) early vascular invasions are common.

Prognosis of HCC, therefore, often depends very much on that of liver cirrhosis, namely a considerable number of patients with early stage of HCC die of liver failure due to accompanying liver cirrhosis. Figure 2 shows the prevalence of hepatitis viral markers in patients with HCC seen in our department from April to November 1991. Only 19% of these were negative for hepatitis viral markers. Liver transplantation in patients with positive hepatitis viral markers, especially HBs antigen, has been proven to generelly have a poor prognosis because of recurrent hepatitis viral infection in donor livers, which usually progress rapidly to the advanced stage.[2]

In addition, vascular invasions into the portal or hepatic vein often occur in the early stage, since extrahepatic metastasis occurs frequently in cases with tumors of HCC over 5 cm in diameter.

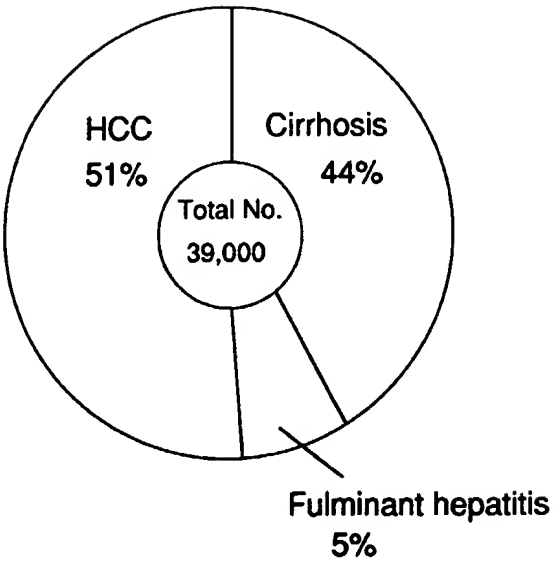


Fig. 1. Number of deaths annually due to liver diseases in Japan

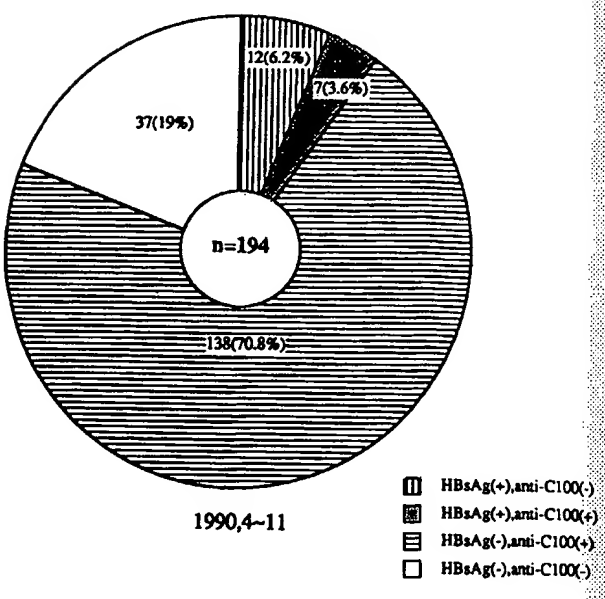


Fig. 2. Prevalence of hepatitis viral markers in patients with HCC seen in the 2nd Department of Medicine, Kurume University from April to November 1991. *HCC*, hepatocellular carcinoma; *LD*, liver disease; *HBsAg*, hepatitis B surface antigen

Table 1. Death from liver diseases in general population

	Death from LD	General population
USA	30	240,000
United Kingdom	4	60,000
Japan	40	120,000
		(thousand)

LD, liver disease

Given these factors, liver transplantation in Japan has numerous problems for consideration. It is therefore meaningful to show the present status of HCC management as carried out in our department as one example for the consideration of liver transplantation in Japan.

Present Status of Diagnosis and Treatment of HCC in Japan

At present, it is possible to detect a tumor as small as 5 mm in diameter by ultrasonography and a definite diagnosis can be easily made by an aspiration biopsy using a fine needle designed by our own department (Majima needle).[3] Since most HCC are found during the clinical course of chronic liver disease, we, therefore, examine patients with liver cirrhosis or chronic hepatitis over 40 years of age every 3 months by ultrasonography to detect small lesions of HCC.

Our previous study indicated that 45 cases (12.1%) of early HCC had been detected in 371 cases of either cirrhosis or chronic hepatitis by ultrasonography, during the follow-up of 421 days on an average, and this means that about 10% of the cases with chronic liver diseases were found to have small HCC in their 1-year follow-up (Fig. 3). This incidence is significantly high, and early HCC cases measuring less than 2 cm in diameter have increased each year in our department.

On our selection of treatments for small HCC less than 3 cm in diameter, surgical resection is indicated if the tumor is solitary and located relatively near the surface of the liver in patients with good liver function (classified as Child A).[4]

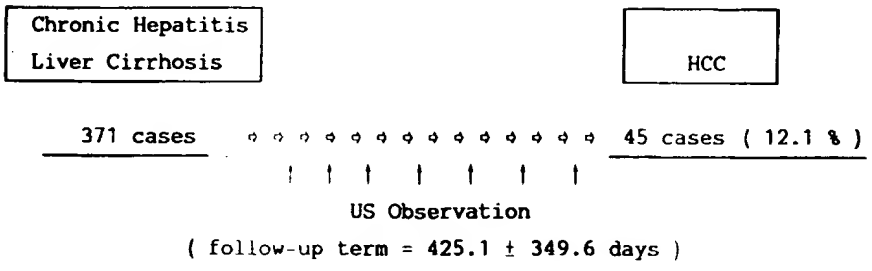


Fig. 3. Detection of HCC in the regular follow-up of chronic liver diseases by ultrasonography in the 2nd Department of Medicine, Kurume University. *HCC*, hepatocellular carcinoma

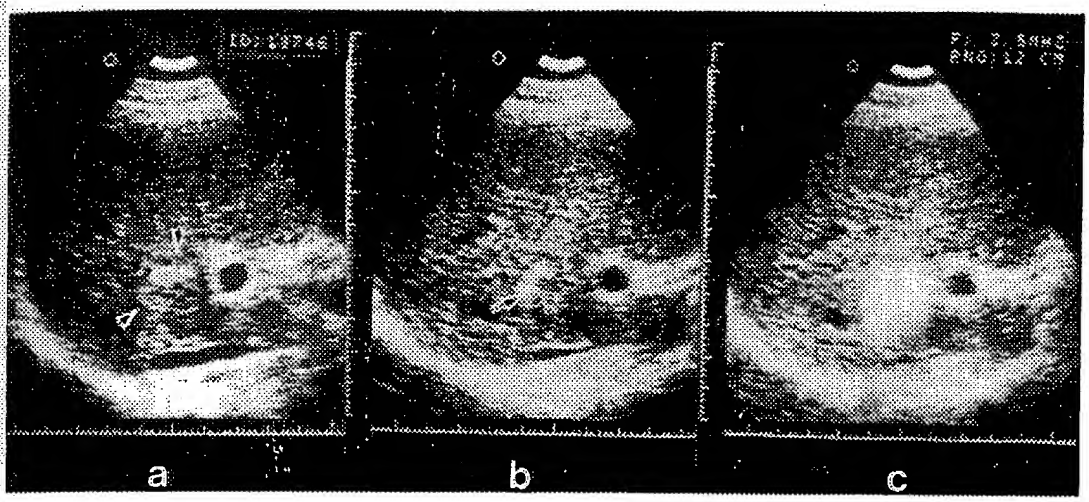


Fig. 4a–c. Percutaneous ethanol injection therapy (*PEIT*) under ultrasonographic guidance. a Small HCC (*arrows*); b The tip of a small needle is observed inserted into the small HCC; c Soon after injection of absolute ethanol. *HCC*, hepatocellular carcinoma

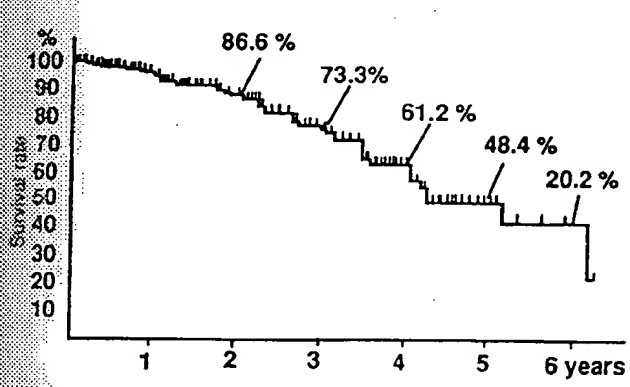


Fig. 5. Survival of 217 cases received percutaneous ethanol injection therapy (*PEIT*) in small HCC less than 3 cm in diameter in the 2nd Department of Medicine, Kurume University (n = 217: alive, 174; dead 43). *HCC*, hepatocellular carcinoma

In cases of small HCC, not indicated for surgical resection, percutaneous ethanol injection therapy (PEIT) is normally carried out in our department.[5] Figure 4 demonstrates an ultrasonogram of the liver during PEIT. The 5-year survival of our 217 patients who received PEIT is 48.4% as shown in Fig. 5. This means that about half of the HCC cases can survive for five years if they are discovered when still as small HCC. Our studies have indicated that the most important factor in the prognosis of PEIT was the cell differentiation of HCC tissue and the prognosis of well-differentiated HCC was significantly better than that of poorly differentiated HCC.[6]

Until now, 43 out of 217 cases treated by PEIT have died and their causes of death were mainly due to hepatic failure in the early period after the PEIT, however death due to malignant extension increased thereafter. Most cases with cancer death were either poorly differentiated in histology or were not well followed clinically after the intitial PEIT.

At present, nodular lesions of HCC over 3 cm in diameter are mainly treated by transcatheter arterial embolization (TAE) combined with an infusion of anticancer drugs except for cases indicated for surgical resection which is rare. The survival curve of our 374 cases treated by TAE is shown in Fig. 6. The 3-year survival rate of 22.2%, is relatively poor compared with small HCC cases treated by PEIT because in most cases, tumors can not entirely be necrotized by TAE alone, and recently TAE-PEIT combination therapy has produced better therapeutic results.[7] In Japan, oral chemotherapy and immunotherapy using biological response modifiers such as OK 432 are normally given for maintenance therapy. Figure 7 shows the survival curves of HCC patients treated with an oral administration of anticancer drugs alone and of those treated by a combination of OK 432 after TAE. A significantly prolonged survival was noted in patients treated with both anticancer drugs and OK 432 after TAE.[7] This indicates the necessity of multidisciplinary treatment in HCC. For far advanced cases of HCC, an arterial one-shot injection of anticancer drugs by Seldinger's method used to be carried out, however at present, an arterial injection of anticancer drugs is more easily and frequently carried out by the use of a totally implantable injection port system placed under the skin, and the prognosis of far-advanced HCC has been improved. As shown in Fig. 8, more than half of such advanced patients can survive for over 1 year.

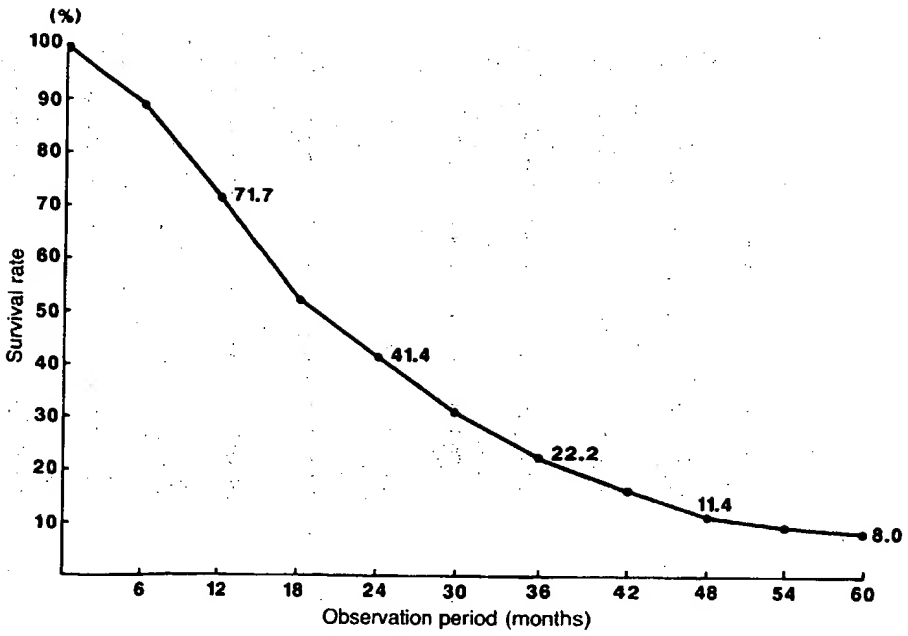


Fig. 6. Cumulative survival rate after transarterial embolization (*TAE*) treatment in 374 cases admitted to the 2nd Department of Medicine, Kurume University (alive, 102; dropped out, 25)

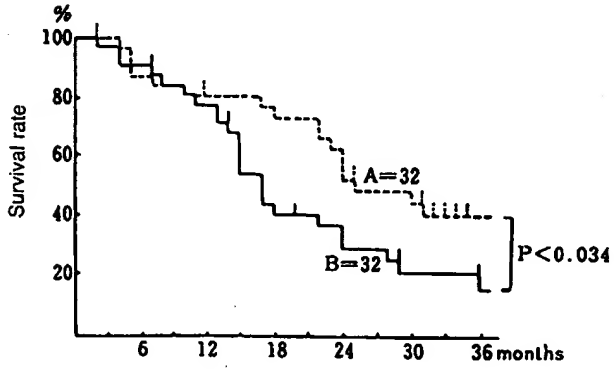


Fig. 7. Survival of HCC patients treated by oral chemotherapy alone (*B*) or with OK432 (*A*) after TAE: 1-year survival, A = 80.5% versus B = 77.7%; 2-year survival, A = 52.5% versus B = 29.6%; 3-year survival, A = 40.9% versus B = 16.2%. *TAE*, transarterial embolization

Table 2. Size of tumor and extrahepatic metastasis in HCC

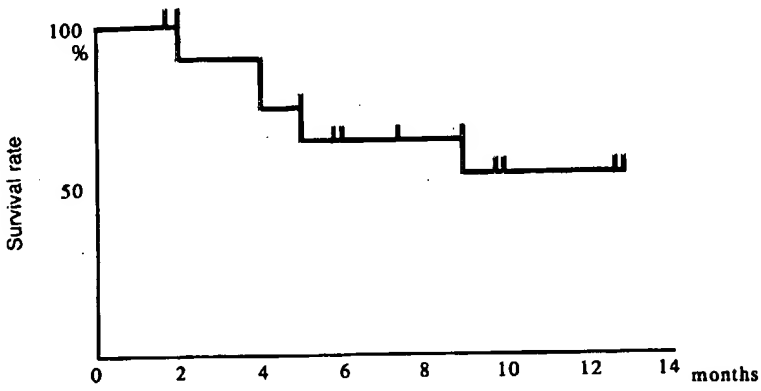
Size of tumor	extrahepatic metastasis (+)	(−)
≤3 cm	2	0
3<, ≤5 cm	5	1
5<, ≤10 cm	2	4
10 cm<	4	23

HCC, hepatocellular carcinoma

Liver Transplantation for HCC at Present in Japan

In advanced HCC, extrahepatic metastasis is common. Our autopsy study showed that extrahepatic metastasis is often seen in HCC tumors over 5 cm in diameter (Table 2). However, it is impossible to detect all of the metastatic lesions by clinical examinations using imaging diagnostic procedures. We could therefore detect only half of the metastatic lesions by imaging diagnostic procedures in comparison with autopsy examination. Figure 9 shows metastatic lesions demonstrated by various imaging methods. Therefore, liver transplantation is not indicated for cases of HCC with extrahepatic metastasis, and a diagnosis of extrahepatic metastasis is extremely important when considering liver transplantation. From the present status of HCC management carried out in Japan, at least four problems arise when in considering liver transplantation:

1. There are numerous patients with HCC in Japan (annual deaths: 20,000) and the number of liver donors is expected to remain small for sometime after the start of liver transplantation in Japan. Thus, it is both difficult and unpractical to select patients with HCC for liver transplantation.
2. The present results of treatment for HCC are considered to be fairly good in Japan. The 5-year survival of patients with small HCC treated by surgery or PEIT is approximately 50%. Small HCC which usually have no extrahepatic metastasis and is theoretically indicated for liver transplantation, would not be realistically indicated for liver transplantation at present in Japan.
3. Over 80% of HCC patients in Japan are HBV or HCV carriers, and the prognosis of liver transplantation in hepatitis viral carriers is not satis-



tumor stage	No.	Vp factor	No.	Vv factor	No.
II	1	Vp 0	4	Vv 0	11
III	2	1	0	1	0
IV-A	13	2	5	2	5
IV-B	1	3	8	3	1

Fig. 8. Effect of arterial chemotherapy using a totally implanted injection port system in far-advanced cases of hepatocellular carcinoma ($n = 17$). *Vp*, Vascular invasion to the portal vein; *Vv*, Vascular invasion to the hepatic vein

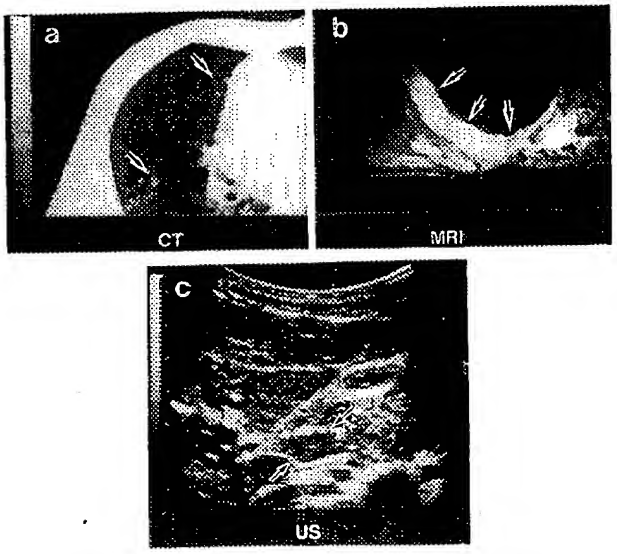


Fig. 9a–c. Imaging diagnosis of extrahepatic metastasis in hepatocellular carcinoma in a lung, b bone, and c lymph node

factory, and thus most HCC patients in Japan are not good recipients.

4. In advanced HCC, at present, we have no good imaging diagnostic procedures which can demonstrate small lesions of extrahepatic metastasis. Generally, HCC cases with no extrahepatic metastasis are indicated for liver transplantation. However, good results can also be obtained by surgery or PEIT for small HCC which usually have no metastasis outside of the liver. In addition, at present, it is impossible to detect small lesions of extrahepatic metastasis in advanced cases.

Considering these facts, liver transplantation appears not to be the first choice of treatment for HCC at present in Japan. However, in the future, it is hoped that HCC can become one of the important hepatic diseases indicated for liver transplantation. In preparing for this, it is necessary to establish effective management techniques to control accompanying hepatitis viral injections and to improve imaging diagnostic procedures in order to detect small extrahepatic metastasic lesions.

In conclusion, at present, liver transplantation is not be the first choice of treatment for HCC, however, in the future liver transplantation may indeed become one of the important therapeutic modalities as transplantation gains in popularity throughout Japan.

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EXPERIMENTAL ENU INDUCED BRAIN TUMORS WITH HpD AND DYE LASER LIGHT

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INTRODUCTION

It is well know that the Hematoporphyrin Derivative (HpD) accumulates preferentially in tumor tissues as compared with the normal tissues (Leonard and Beck, 1971; Lipson et al., 1961; Dougherty et al., 1976).

A sizeable reduction in tumor mass for a number of different types of non-neural tumors was obtained by the irradiation of the tumor mass with light in animals that had received HpD at the doses of from 2 to 5 mg/kg of body weight (Dougherty et al., 1975, 1978; Gomer and Dougherty, 1979).

Diamond et al. (1972) demonstrated that glioma cells, which are grown in cell culture or implanted subcutaneously in rats, could be destroyed by exposure to laser light if they had been photosensitized previously by treatment with Hematoporphyrin.

Tumor necrosis induced by HpD photosensitization seems to function on a basis of resonant energy transfer between triplet HpD and the endogenous molecular oxygen. After the HpD molecules that accumulate in the tumor are brought to their first excited singlet state by the absorption of light, most of them decay to the long-lasting triplet state. When triplet HpD collides with an oxygen

molecule, the oxygen molecule is excited to its first or singlet state. The singlet oxygen produced by this photodynamic action, is known to be radical and is likely to be the cytotoxic agent responsible for this tumor necrosis. The most effective results of this tumor phototherapy are achieved using red light (631 nm wavelength) at doses of 100 J/cm^2.

MATERIALS AND METHODS

Tumor Induction

A single dose of Ethylnitrosourea (ENU) (20 mg/kg i.v.) was given to pregnant Fisher-344 rats on the 16th day of gestation according to Grossi-Paoletti et al. (1972).

A systematic diachronic study of the brain was begun on the 15th day of extrauterine life (Shiffer et al. 1978, 1980).

Hematoporphyrin Determination

Hematoporphyrin was extracted from tissues and then measured according to a modified procedure of Lemberg and Legge (Spanu et al., 1983).

The Hematoporphyrin concentration in the HCl solution was determined with a Perkin-Elmer MPF 4 spectrophotofluorimeter. An estimate of the amount of Hematoporphyrin was obtained by calculating the sum of the emission intensities at 630 nm, proportional to the integrated area of the emission spectrum in the 550-750 nm wavelength range and amount of Hematoporphyrin in 1M HCl solution (Jori et al., 1979).

Laser Irradiation

Sixty animals were divided into three groups and, starting on the 90th day of extrauterine life, treated as follows: one control group (Group 1) underwent laser treatment only, the second control group (Group 2) underwent combined treatment with HpD + laser, while the group treated with ENU (Group 3) also underwent combined treatment with HpD + laser. The Hematoporphyrin Derivative (Photofrin, 5 mg HpD per ml) was administered i.p. at a dose of 5 mg/kg. All animals were irradiated 48 hours after HpD injection. Each group received 2 laser treatments 3 days apart.

Ten animals of each group were sacrificed 24 hours after the first treatment and the remaining animals were sacrificed 15 days after the second treatment.

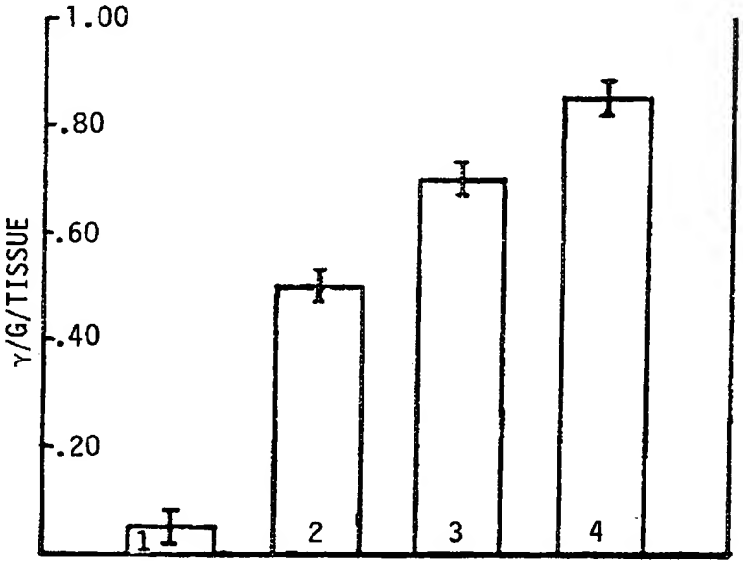
Carnoy at 0° was used to fix the brains which were then dehy-

drated, paraffin embedded and cut into 4 μm thick serial sections. The following stains were employed: hematoxylineosin (HE), PTAH, luxol fast blue B for myelin.

The following zones were examined with progressing development: germinal zone, cortex, mantle zone, basal ganglia, medullary center, paraventricular white matter and subependymal plate. Cell count was performed in microscope fields of 160 μm^2 x 110 μm^2.

The irradiation source was cw (continuous wave) dye laser (Coherent 599) pumped by Argon ion laser (Coherent CR-18). The lasing dye Rhodamine B (Exciton Rhodamine 610) dissolved in Ethylglycol was used. The dye-laser output was split into two beams of the same power and coupled into two optical fibers of 400 micron diameter. A craniotomy of 2 mm in diameter was performed and each single fiber was placed on the dura using a sterotaxic device. All irradiation was performed using 200 mW per fiber for 10 minutes.

Table 1. Uptake of HpD in Normal and Tumoral Brain



1 = NORMAL TISSUE; 2 = ISOMORPHIC OLIGODENDROGLIOMA; 3 = POLIMORPHIC OLIGODENDROGLIOMA; 4 = GASSER NEURINOMA

EACH VALUE REPRESENTS THE MEAN ± S.E.M. OF LAST FIVE DETERMINATIONS.

RESULTS

The uptake values of HpD in the brain tissues of the control rats in different oncotypes induced with ENU are shown in Table 1. The values referring to normal brain tissues present a much lower uptake of HpD. We can consider this value equal to zero since if refers to tiny traces of substances still present in the blood and in the capillaries included in the tissue homogenate. Contrarily, the values found in the three oncotypes evaluated are at least 5 times higher than in the controls: isomorphic oliogodendroglioma incorporates .50 gamma/gr of tissue, polymorphic oliogodendroglioma .72 gamma/gr and neurinoma .81 gamma/gr.

Six animals died between 6 and 12 hours after the second application of the laser. In 2 cases, the cause of death was attributed to an intracranial hematoma and in 4 cases to a diffuse brain edema.

Histological Observation

In the rats sacrificed 24 hours after the first laser treatment, in proximity of the hole of the craniotomy, there was a non-delimited necrosis, characterized by eosin pallor and nuclear phyknosis of the cortex and while matter.

As for the animals sacrificed 15 days after the second laser treatment, Group 1 showed a necrotic cyst surrounded by a moderate gliomesodermic reaction; the lesion was situated in the cortex and subcortical white matter close to the point of laser application.

In the rats of Groups 2 and 3, localization and size of the necrosis were approximately the same as in Group 1; the most striking difference was the intensity of the gliomesodermic reaction, consisting of abundant macrophages, vessel proliferation with endothelial hyperplasia, and hypertrophic reactive astrocytes. Mitotic activity was prominent, both in the reactive elements and in the immediately surrounding glial cells.

We had no chance to observe laser effect on tumors, since the tumor lesions were very small at the age of 90 days.

CONCLUSIONS

In the 60's and 70's various authors demonstrated the accumulation of HpD in tumoral tissues (Leonard and Beck, 1971; Lipson et al., 1961, Weishaupt et al., 1976; Winkelman and Rasmussen-Taxdal, 1960; Carpenter et al., 1977, Dougherty et al., 1976). Moreover, other authors demonstrated the distribution of this substance in the tissues of various animal species (Dougherty et al., 1975, Gomer et al., 1979; Laws et al., 1981; Rounds et al., 1982). Although

there is little data from the literature regarding HpD determination in spontaneous brain tumors, various studies have attempted to determine the presence of HpD in subcutaneously transplanted glial tumors.

Our data on brain tumors, which were induced with ENU, show a significant accumulation of HpD in different oncotypes. This data are similar to the preliminary experiments conducted on man using fluorescence to determine HpD by Perria et al., 1980; and Laws et al., 1981.

In the cases presented by Perria et al., three of which underwent both bioptic and autopsic histologic diagnosis after treatment, the authors found necrotic areas near the tumor which they relate to photoradiation treatment.

In the cases reported by Laws et al., which were only investigated by CT scan, wide necrotic-hemorrhagic-cystic cavities were shown.

Our data, on the other hand, which refers to a limited period of observation, show that the use of HpD + laser in this experimental mode with this technique, tends to stimulate a very marked cellular response with respect to tissue damage as the quantity of mitoses in the reaction tissues demonstrates. The biologic potential of this intense proliferative activity caused by this treatment requires further investigation and an evaluation of its effects after longer periods of time. It is difficult to compare histological data reported up to now because of the difference in the intervals at which the determinations were performed.

In order to evaluate the efficacy of this treatment, in our opinion, it is necessary to perfect the parameters used (duration and potency of therapy, exposition time, determination of itracellular location of HpD) and to learn more about the mechanism of cell destruction. These are open problems which must be solved at an experimental level before this treatment, which must still be proved to be effective in the therapy of brain tumors, may be applied in man.

SUMMARY

The authors describe HpD determination in normal brain tissue and in experimental brain tumors induced by ENU in rats. In the same study the animals were treated with laser irradiation (HpD + dye laser).

Histological evaluation were made of the following: 1) the effects of the therapy with laser light only in animal controls, 2) the effects of HpD + laser in another group of animal controls,

and 3) the effects of HpD + laser in a group of animals with tumors induced by ENU.

The histologic evaluations refer to a brief period of observation; therefore data on tissue alterations caused by HpD + laser cannot be considered definitive.

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STUDIES ON SOME BIOLOGICAL FUNCTIONS OF MACROPHAGES ACTIVATED BY HeNe LASER PHOTODYNAMIC TREATMENT AS COMPARED TO *CORYNEBACTERIUM PARVUM* AND INTERFERON ACTIVATION

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Some biological properties of macrophages activated with *Corynebacterium parvum*, recombinant murine interferon-gamma (rMuIFN-γ) and HeNe laser photodynamic therapy (PDT) were studied. Results obtained indicate the following: (i) macrophages activated with the three immunopotentiators have an intense phagocytic activity; (ii) the association of PDT-treated T and B peritoneal lymphocytes with macrophages increases their biological properties; (iii) the cytotoxic activity of photodynamically - activated macrophages (PD - AMϕ) varied between 42.8 and 59.4% of the normal function of the tumour target cells; and (iv) the three sets of macrophages exert cytostatic effects upon human and mouse leukaemia cells. In conclusion, macrophage immunopotentiation with photodynamic therapy has proved to be a useful method in cellular biological studies as well as for adoptive immunotherapy in various cancer forms.

KEY WORDS Photodynamic therapy Photofrin II He-Ne laser Activated macrophages *Corynebacterium parvum* Recombinant murine interferon-gamma

Introduction

Biological activities (bacteriocidal, cytotoxic and cytostatic) of macrophages were stimulated by a variety of agents such as: *C. parvum*, interferon, phorbol myristate acetate (PMA), lipopolysaccharide (LPS), the late complement component C5-9 and mediated by numerous products, such as TNF-α, neutral proteases, metabolic and oxygen.[1–4]

During activation of macrophages, reduction of oxygen produces toxic agents such as the superoxide anion (0_2), hydrogen peroxide (0_2H_2), the hydroxyl radical (0H') and singlet molecular oxygen ($^10_2$).[5]

In the last few years successful attempts have been made to demonstrate macrophage activation and the increase of the membrane permeability (for calcium ions) after irradiation with light of specific wavelengths and different energy densities.[6–8]

Recently, it has been proved that activated murine macrophages can induce regression of murine tumours and pulmonary metastases.[9] Similarly, human and murine macrophages differentiated from blood monocytes and activated by interferon-gamma have been used in adoptive immunotherapy.[10–12]

The present study reports on the biological functions of macrophages activated by HeNe laser therapy (HeNe PDT) as compared to activation by *C. parvum* and interferon.

Materials and Methods

Activation of macrophages (AMϕ) was achieved by three methods:

(1) Monocytes Isolated from Rat Blood by the Method of Bartholeyns et al.[10]

Briefly, peripheral blood monocytes were isolated by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and were obtained with a purity of 62–67%. These monocytes were cultured in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (50 μg/ml), oxalacetic acid (132 μg/ml), insulin (8 μg/ml), glutamine (2 mM, pyruvic acid (2 mM) 5×10^{-6} M indomethacin, non-essential amino acids (1 ml Gibco concentrate /

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100 ml) and 10% fetal calf serum (Gibco-Europe, U.K.). Monocytes were grown in Pyrex bottles (up to 10^6 cells/ml) at 37°C in a humidified 5% CO_2/95% air incubator. Cells differentiated into macrophages after 6 days of culture, as shown by their adherence and the presence of non-specific esterases. The effector cells were activated by exposure to 500 units/m of recombinant murine interferon-γ (rMuIFN-γ, 5×10^6 U/mg, Genetech Inc., South San Francisco, U.S.A.) for 24 h. This group is hereafter referred to as IFN-AMϕ.

(2) *Activation by* Corynebacterium parvum

A suspension of killed *C. parvum* (Wellcome Research Laboratories, Beckenham, Kent, England) was used for intraperitoneal immunization of rats (20 mg/kg) to induce activation of the biological functions of macrophages after 7 days. Exudate cells harvested by injecting 20 ml of Eagle medium containing 10 units of heparin/ml into the peritoneum. The abdomen was massaged for a few minutes and the peritoneal fluid was then withdrawn with a 21 gauge needle attached to a 21 ml syringe. The *C. parvum*-AMϕ cells were then washed three times with phosphate buffered saline (PBS) and resuspended in phenol red-free Eagle's medium with 10% fetal calf serum.

(3) *Isolation and Photobioactivation of Macrophages*

Rats were intraperitoneally inoculated with 10 ml paraffin oil; 96–120 h later, the peritoneal exudate was extracted and prepared as above for *C. parvum*-AMϕ. The viability was evaluated by the trypan blue test (0.3% solution). Macrophages were suspended in Eagle's medium (phenol red-free containing Photofrin II and, having received HeNe laser irradiation, were incubated for 60 min at 37°C in the dark. Subsequently, the PD-AMϕ cells were washed three times with PBS, resuspended in RPMI 1640 supplemented with 10% fetal calf serum and used for evaluating phagocytic, cytotoxic and cytostatic biological activities.

Photosensitizer

Photofrin II (Photofrin Medical Inc. Cheetkowage, N.Y.) was used to sensitize rat peritoneal macrophages with doses in the range of 0.01–10.0 μg/106 cells.

Laser Phototherapy

Our light source was a divergent beam from a LG HeNe laser (632.8 nm; 8 mW) whose output was checked periodically with a Spectraphysics C powermeter. Photofrin II sensitized macrophages were HeNe laser irradiated at three dose fluences: 1.5, 4.5, and 9.0 kJ/m^2.

Diabetes Mellitus

Diabetes mellitus was induced in inbred Wistar-R8 rats by inoculating them with alloxan (120 mg/kg body mass) intraperitoneally. Ten days later peritoneal lymphocytes (T and B) were isolated and prepared according to the method described by Kol *et al.*[13]

The degree of macrophage activation was estimated by means of the following parameters: (a) phagocytosis of *E. coli* bacterial cells labelled with ^{3}H-thymidine; (b) cytotoxic and cytostatic activity against leukaemia target cells; (c) observation with the transmission electron microscope (TEM).

Phagocytizing Procedure. The enterotoxigenic strain of *Escherichia coli*-H 10407, serotype 078:H11 (provided by Professor Dr M. M. Levine, University of Maryland, Baltimore, U.S.A.) was cultivated on Evans medium[14] and labelled with ^{3}H-thymidine according to the method described by Dima *et al.*[15] Equal volumes (100:1 ratio) of suspensions of bacterial cells (1×10^7 cells/ml) and PDT activated macrophages (1×10^5 cells/ml) were incubated for 3 h at 37°C. After incubation, macrophages were isolated and washed three times with PBS by centrifugation at 1200 rpm/10 min, filtered through Millipore membrane (ϕ; 0.45 nm) and sealed in vials containing 0.4% PPO (2,5-diphenyloxazole) and 0.01% POPOP (1,4 bis-(5-phenyloxazolyl) benzene in toluene. Phagocytosis of labelled bacteria was expressed in percentages against the values found in the control (considered as 100%).

Tumour Target Cells. The following cell lines were used: K562 (human myeloid tumour cells); MOLT-4 (human leukaemia T cells); YAC-1 (mouse T cell lymphoma) and RL♂1 (mouse leukaemia). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum.

Cytotoxicity Assay. Cytotoxicity of the effector cells was assayed by the ^{3}H-uridine method as described previously by Nishimura *et al.*[16] Briefly, aliquots (0.1 ml) of effector cell suspensions were mixed with 0.1 ml of ^{3}H-uridine (spect. act. 30 Ci/mmol, IFA Bucharest, Romania) labelled target cells (5×10^4/0.1 ml) in Coster wells of round-bottom plates. Rat activated macrophages (IFN-AMϕ; *C. parvum*-AMϕ; PDT-AMϕ) were added to the target cells at several effector to target ratios (12.5:1 to 100:1) and incubated for 18 h at 37°C. The cells were harvested with a harvester and the retained radioactivity determined by a standard

scintillation technique. Target cell lysis was calculated by the following equation:

$$\% \text{ cytoxicity} = \left[\frac{1 - \text{cpm in culture of effector and target cells}}{\text{cpm culture of target cells alone}} \right] \times 100$$

All tests were set up in triplicate. The background isotope release over 18 h was less than 12.5%.

Cytostatic Activity. Cytostatic activity of normal macrophages (NMϕ) and of those activated with PDT, *C. parvum* and rMuIFN-γ were tested and calculated in percentages by the method described by Tsuchiya *et al.*[17] Macrophages (2×10^6) were cultured *in vitro* with 2×10^4 leukaemia cells (K 562, MOLT-4, YAC-1, RLσ1) in 0.2 ml RPMI 1640 medium, supplemented with 10% fetal calf serum. The solution was incubated in wells of flat-bottomed microtitre culture plates at 37°C for 48 h in 5% CO_2 atmosphere. Cells were harvested with a harvester. Eighteen hours before radioactivity determination, 0.1 μCi tritiated thymidine was added to every well. Radioactive precursor incorporation (^{3}H-TdR) into tumour cells measured by a liquid scintillation counter (Beckman). Cytostatic activity of activated macrophages was expressed as percentage of the inhibition of ^{3}H-TdR incorporation into the leukaemia cells.

Electron Microscopy. After 3 h incubation at 37°C, the normal and activated macrophages–bacteria mixtures were twice washed with PBS and the sediment was fixed for 1 h in 2.5% glutaraldehade (0.1 M, pH 7.2); post-fixed for 60 min in 1% OsO_4 (0.15 M, pH 7.2) and embedded in Vestopal. These sections were obtained with an ultramicrotome Porter-blume-MT 1. Uranyl acetate and lead citrate staining was performed. A 75 kV Hitachi-HLL electron microscope was used.

Statistics

Data were expressed as the arithmetic mean + the standard error (SE). The statistical significance of differences between groups was calculated by the Kruskal-Willis test.

Results

Photodynamic Activation of Peritoneal Macrophages

The results presented in Figure 1 reveal an activation of rat peritoneal macrophages following sensitization with different Photofrin II concentrations (0.01–10.0 μg/10^6 cells) and exposure to various

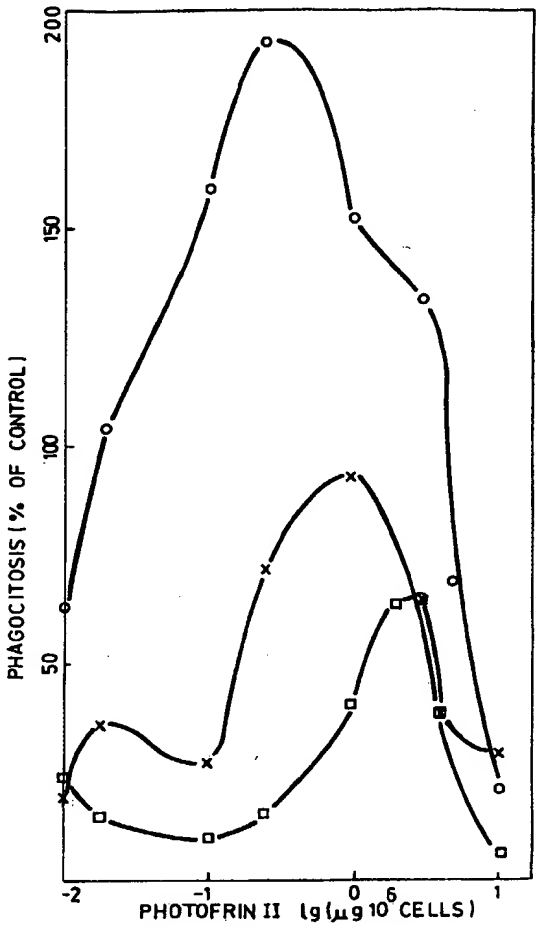

Figure 1. Activation of macrophages by photodynamic therapy. Phagocytosis was estimated at various Photofrin II concentrations (0.01 to 10.0 μg/10^6 cells) and at a fluence of: -o-o- 1.5 kJ/m²; -x-x- 4.5 kJ/m²; and -□-□- 9.0 kJ/m² of HeNe laser irradiation

HeNe laser doses (1.5–9.0 kJ/m²). The highest rate of bacterial ingestion (192.3%) was found in Photofrin II-sensitized macrophages at a concentration of 0.5 μg/10^6 cells and exposure for 30 s to HeNe laser irradiation (1.5 kJ/m²). As the HeNe laser irradiation dose increased (4.5 and 9.0 kJ/m², respectively) a rightward deviation of the Photofrin II concentration necessary to sensitize macrophages (Figure 1) was noticed.

Macrophage sensitization with higher Photofrin II concentrations (2–10 μg/10^6 cells) and prolonged exposure to HeNe laser irradiation doses (4.5 and 9.0 kJ/m², respectively) induced partial inhibition of the phagocytic activity of macrophages at lower values (41.7%) than those found in non-treated macrophages (Figure 1).

Phagocytosis of *E. coli* bacteria by macrophages activated *in vivo* and *in vitro* with *C. parvum* (285.7%) and rMuIFN-gamma (238.9%) was higher

Table 1. Activation of macrophages by photodynamic therapy, interferon and *Corynebacterium parvum*

Treatment	Phagocytosis % 3 h at 37°C
PDT* – AMϕ†	192.5
rMuIFN-gamma‡ – AMϕ	238.9
C. parvum – AMϕ§	285.7

* PDT = Photodynamic therapy.
† AMϕ = Activated macrophages.
‡ rMuIFN-gamma = Recombinant murine interferon-gamma.
§ *C. parvum*-AMϕ = *Corynebacterium parvum* activated macrophages *in vivo*.

than the values found in PDT-activated macrophages (Table 1).

These observation point to the existence of a correlation between macrophage biological activities and Photofrin II concentrations on the one hand and the HeNe laser irradiation dose on the other.

Cocultivation of Photodynamically Treated T and B Lymphocytes with Macrophages

Rat peritoneal (T and B) lymphocytes were suspended in Eagle's medium (phenol-red-free) containing 0.5 μg/m Photofrin II and, after irradiation with 1.5 kJ/m2 HeNe laser, were incubated for 60 min at 37°C in the dark. Subsequently, the lymphocytes were washed three times with Eagle medium and mixed with peritoneal macrophages in RPMI-1640 medium + 10% fetal calf serum and reincubated at 37°C with bacteria (labelled with ^{3}H-thymidine) for bacterial ingestion.

The phagocytic capacity of macrophages increased significantly after 3 h at 37°C. Moreover, it could be noticed that bacterial ingestion increased when the mixture was obtained using T and B lymphocytes from animals stimulated *in vivo* with *C. parvum*. In contrast, microbial ingestion showed a decline (42.3%) when macrophages were mixed with T and B lymphocytes from animals with alloxan-induced diabetes mellitus (Table 2).

Table 2. Cocultivation of T and B lymphocytes treated photodynamically with macrophages

Treatment	Phagocytosis (%) 3 h at 37°C
PDT* – AMϕ†	234.7
NMϕ + PDT – T-B cells‡	312.3
C. parvum-AMϕ + PDT – T-B cells	363.1
NMϕ§ + PDT – T-B cells (DM)‖	42.3

* PDT = Photodynamic therapy (Photofrin II 0.5 μg/10^6 cells + 1.5 kJ/m^2 HeNe laser).
† AMϕ = Activated macrophages.
‡ T-B cells = Normal peripheral T and B lymphocytes.
§ NMϕ = Normal macrophages.
‖ T-B cells (DM) = Diabetes mellitus peritoneal T and B lymphocytes.

Cytotoxic Activity of Activated Macrophages

The results presented in Figure 2 point out the capacity of PDT to stimulate the cytotoxic activity of macrophages against target cells (K562, MOLT-4, YAC-1, RL♂ 1) between 42.8% and 59.4%.

PDT-activated macrophages showed higher values against YAC-1 and RL♂ 1 and relatively lower values against K562 and MOLT-4 target cells in comparison with non-stimulated cells (values in the range of 18.5% and 23.9%).

C. parvum-activated macrophages showed higher cytotoxic values against MOLT-4 and YAC-1 target cells and medium compared with RL♂ 1 and K562.

In PDT and rMuIFN γ activated macrophages one higher cytotoxicity value was noted for the murine leukaemia lines (RL♂ 1 and YAC 1) and relatively moderate for the human leukaemia lines (MOLT-4 and K562) (Figure 2).

The lysis of target cells was estimated after 18 h at 37°C and gradually increased according to the effector to target ratio (12.5 to 100:1). Macrophage cytotoxicity was suppressed after sensitization with high Photofrin II doses and longer exposure to HeNe laser irradiation (data not shown).

Cytostatic Effects

The cytostatic effects of macrophages activated with PDT, *C. parvum* and rMuIFN-γ were studied on four human and murine leukaemia lines and the values found after 18 h of incubation at 37°C were analysed.

The results obtained on the cytostatic effect of activated macrophages allowed of the following observations: (i) all the three sets of macrophages had a cytostatic activity ranging between 44.3 ± 2.1 and 82.0 ± 4.7%; (ii) of the three sets the most marked cytostatic activity was that of *C. parvum*-AMϕ against three (K562, RL♂ 1 and YAC-1) of the four leukaemia lines and relatively low against MOLT-4; (iii) PDT AMϕ had a more significant cytostatic activity against K562 and YAC-1 and more reduced against RL♂ 1 and MOLT-4; (iv) the cytostatic activity of rMuIFN-γ-AMϕ was quite similar to that found for *C. parvum*-AMϕ and PDT-AMϕ as against the control (untreated) cells (Table 3).

These observations suggest that macrophages activated with all the three immunopotentiators induce marked cytostatic effects and that complete cytostasis is not obtained in the range of the effector to target ratio (100:1) used in the present study.

Electron Microscopy

Normal macrophages and macrophages activat d by different immunostimulators were submitted to

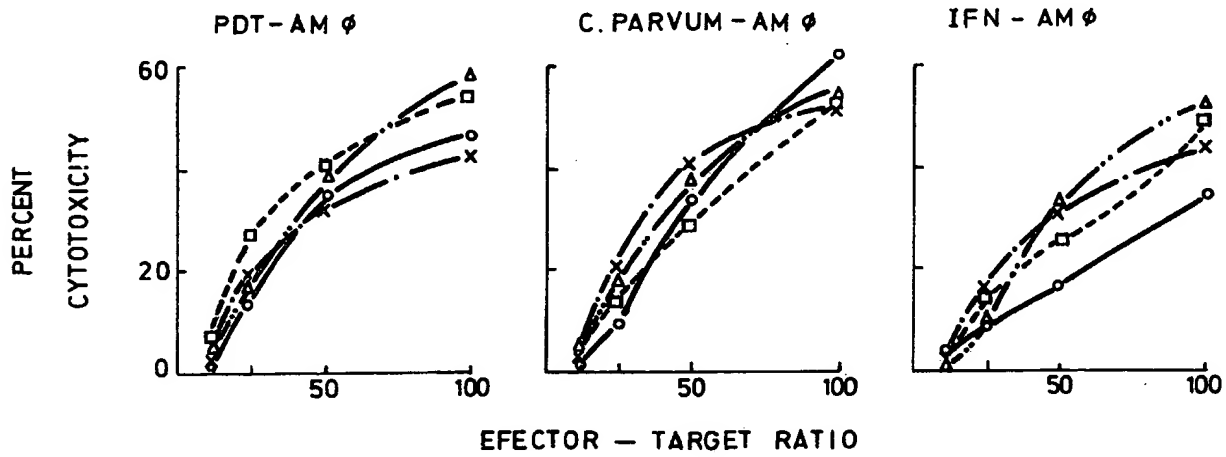

Figure 2. Tumour cell sensitivity to activated macrophages. Cytotoxic activity was measured in 18 h assay at different EC/TC ratios. Spontaneous release of the isotope from target cell was less than 12.5%. -x-x- (K562); -O-O- (MOLT-4) -Δ-Δ- (YAC-1); and -□-□- (RL♂ 1)

Table 3. Comparison of cytostatic activity of macrophages activated by photodynamic therapy, *C. parvum* and interferon

Target Cells	Growth inhibition* (%)		
	PDT	*C. parvum*	Interferon
MOLT-4	55.0 ± 2.6†	53.6 ± 3.1	61.5 ± 3.3
RL♂ 1	44.3 ± 2.1	78.2 ± 4.2	52.7 ± 4.0
K 562	58.5 ± 3.0	69.4 ± 3.9	64.3 ± 3.8
YAC-1	70.1 ± 4.4	82.0 ± 4.7	76.8 ± 4.6

* Cytostatic activity of activated macrophages was expressed as percentage of the inhibition of ^{3}H-TdR incorporation into target cells.
† Mean ± S.D. of three experiments.

incubation for 3 h after *E. coli*-H10407 innoculation; the morphological changes were studied with the electron microscope. Experimental observations are presented in Figures 3–6.

Macrophages obtained from rats activated with *C. parvum*, rMuIFN-γ and PDT exhibited a significantly higher phagocytic activity when incubated 3 h at 37°C with *E. coli* (1:100 ratio).

On electron photomicrographs of PDT-activated macrophages, Golgi apparatus, mitochondria and lysosomes could be observed in cytoplasm (Figure 3).

Figure 4 shows the phagocytic activity of PDT-activated macrophages after 3 h at 37°C. In this figure, the existence of a relative increase of phagocytosed bacteria presenting various degrees of alterations can be noticed. The figure illustrates the elements of the phagocytic process: (a) phagosomes with ingested bacteria: (b) phagocytic vacuoles; (c) presence of amorphous material in phagosomes; and (d) the existence of a space between the phagosome membrane and the bacterial wall. Figure 5 shows the presence of phagocytosed

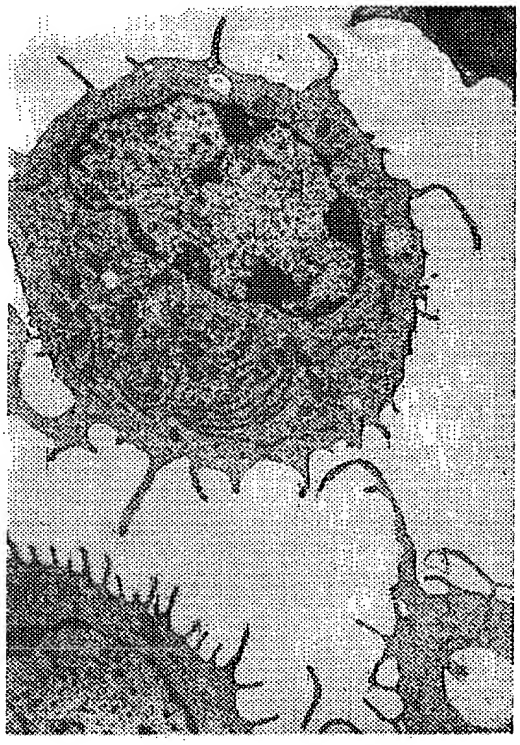

Figure 3. Macrophage activated by photodynamic therapy (0.5 μg/10^6 cells Photofrin II and HeNe laser irradiation 1.5 kJ/m^2). Uranyl acetate and lead citrate staining. Magnification, × 9600

bacteria but without lytic lesions of the bacterial wall and cytoplasm and were observed mostly in *C. parvum*-activated macrophages.

In macrophages sensitized with high Photofrin II doses (3–10 μg/ml) and prolonged exposure to the HeNe laser irradiation (4.5 and 9.0 kJ/m^2) the

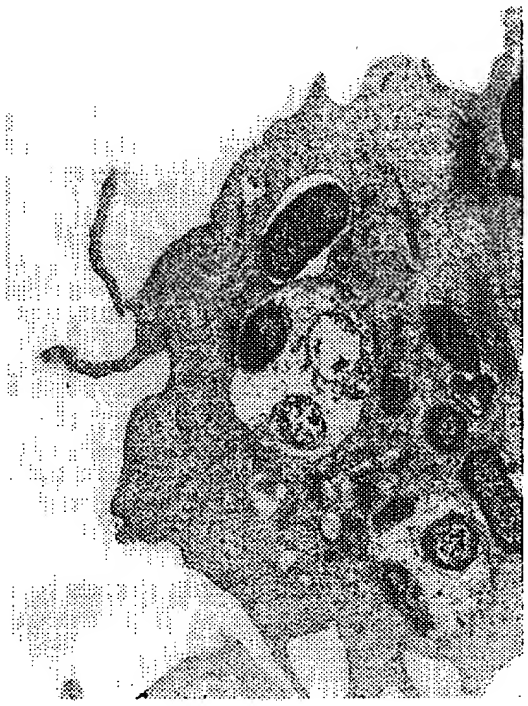

Figure 4. Macrophage activated by PDT, after 3 h incubation at 37°C with *E. coli* - H10407 strain. Uranyl acetate and lead citrate staining. Magnification, × 12 800

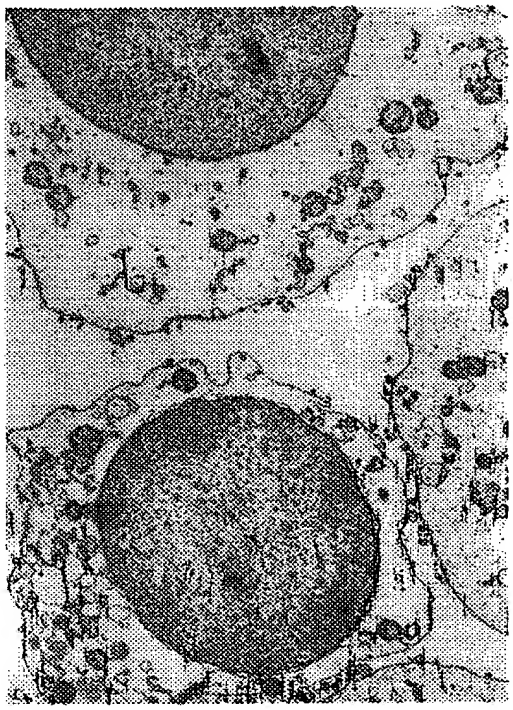

Figure 6. Rat macrophage after sensitization with high Photofrin II doses (5 μg/ml) and longer exposure to HeNe laser irradiation (9.0 kJ/m²). The nuclear membrane is preserved, but chromatin structure and subcellular organelles are seriously damaged. Magnification, × 9000

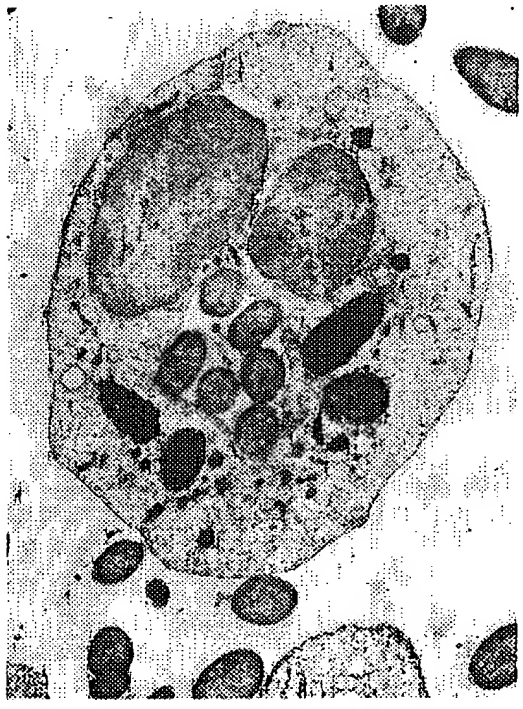

Figure 5. Macrophage activated by *C. parvum*, after 3 h incubation at 37°C with *E. coli* - H10407 strain. Uranyl acetate and lead citrate staining. Magnification, × 10 800

following morphological modifications were noticed: (i) partial preservation of nuclei; (ii) cytoplasmic vacuolization due to lysosome disruption; and (iii) lesions of subcellular organelles (mitochondria, lysosomes and ribosomes) (Figure 6).

Discussion

In the present paper, the activation of rat peritoneal macrophages with photodynamic therapy has been noted to stimulate their bactericidal, cytotoxic and cytostatic functions. The phagocytic activity of PDT-AMϕ, varied in terms of two factors: (a) Photofrin II concentrations used and (b) HeNe laser irradiation dose. Prolonged exposure to HeNe laser irradiation and high Photofrin II doses induced a very noticeable decrease in PDT-AMϕ biological properties with immunosuppressing effects.

Association of B and T peritoneal lymphocytes to macrophages increased their biological properties, whereas addition of lymphocytes from animals with diabetes mellitus produced partial inhibition of macrophage activities.

The cytotoxic and cytostatic values found (expressed in percentages) against target leukaemia cells (K562, MOLT-4, RL♂ 1 and YAC-1) at

photobioactivated macrophages with PDT were appreciably closer to those stimulated by *C. parvum* and rMuIFN-γ. Examination with the transmission electron microscope confirmed the results obtained by means of other methods.

The results obtained are in agreement with the arguments of some authors[18,19] concerning the role of T and B lymphocytes in activating macrophages: we bring further experimental evidence regarding the stimulation of other biological (cytotoxic and cytostatic) functions.

It is well known that *C. parvum* and interferon-activated macrophages have tumouricidal and microbiocidal activities.[20–24] These results allowed two observations: (1) importance of lymphocytes in macrophage activation and (2) their functional, immunologic and metabolic state.

The mechanisms by which macrophages are PDT-activated have yet to be described and we suggest: (a) oxidation of unsaturated lipids which causes an increase in the permeability of the membrane;[25–27] (b) an increase in the fluidity of lymphocyte membrane lipids after photodynamic treatment appears to initiate activation of macrophages for enhanced Fc receptor-mediated ingestion;[28,29] and (c) the visible red light emitted by the HeNe laser has a powerful PDT effect on activation of macrophages sensitized *in vitro* with Photofrin II.[30]

The second remark could be explained by the fact that rats with diabetes mellitus are lymphopenic[31] and have reduced CD5+ and CD4+ cells.[32, 33] They are deficient in: (a) the phenotypic (CD8+/CD5−) and functional expression[34,35] of cytotoxic/suppressor T lymphocytes; and (b) B cells[36] and the phagocytic cells themselves.[37]

Conclusions

The biological functions (bacteriocidal, cytotoxic and cytostatic) of PDT-photobioactivated macrophages were as efficient as those immunopotentiated by *C. parvum* and rMuIFN-γ; macrophages activated with photodynamic therapy have proved to be a useful method for cell biological study as well as for adoptive immunotherapy in various cancer forms.

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Laser-photosensitizer assisted immunotherapy: a novel modality for cancer treatment

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Abstract

Photosensitizer-enhanced laser treatment, where dyes are activated in situ by lasers of appropriate wavelengths, provides highly selective tissue destruction, both spatially and temporally, through photophysical reactions. Although laser-sensitizer treatment for cancer can achieve a controlled local tumor cell destruction on a large scale, total tumor eradication may not be accomplished because of the incomplete local tumor killing or the presence of tumor metastases, or both. The long-term control of cancer depends on the host immune surveillance and defense systems in which both cell-mediated and humoral responses are critical. In this study we report a novel minimally invasive cancer treatment combining the laser photophysical effects with the photobiological effects. Irradiation of a rat mammary tumor by an 805 nm diode laser, after an intratumor administration of a specific photosensitizer, indocyanine green in a glycated chitosan gel, caused immediate photothermal destruction of neoplastic cells. Concomitantly this treatment stimulated the immunological defense system against residual and metastatic tumor cells. Increases in survival rate and in the eradication of tumor burden, both primary and metastatic, were observed after this treatment. Furthermore, the resistance of successfully treated rats to tumor rechallenge demonstrated a long-lasting systemic effect of the treatment. These findings indicate that our treatment has triggered a specific humoral immune response in the tumor-bearing rats. © 1997 Elsevier Science Ireland Ltd.

Keywords: Indocyanine green; Glycated chitosan; 805 nm diode laser; Laser-assisted immunotherapy; Cancer treatment; Humoral immune responses

1. Introduction

The use of lasers in lesion treatment utilizing photosensitizer-enhanced reactions [1,2], particularly for the destruction of malignant tissues, is gaining widespread acceptance because of the precision of energy delivery achieved with modern instruments. When a photosensitizer of appropriate absorption peak is present in the tumor, the laser energy can be directed and deposited in the targeted tissue to cause enhanced and localized photomechanical, photochemical and photothermal reactions [3–5]. This methodology provides a non-invasive treatment modality that minimizes col-

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lateral tissue damage, both local and systemic [1,2,6–8]. However, the very circumscribed effect of this procedure may also limit its overall efficacy, since local treatment could not stop the global neoplastic proliferation that occurred prior to treatment, that evolved from the surviving cells, or both. Therefore, in order to achieve a long lasting effect, it is highly desirable for the localized laser effect to be coupled with a superimposed laser-induced systemic photobiological reaction.

A novel approach which takes advantage of the precise localization of the laser thermal tumor destruction and at the same time stimulates the host's immune defense system has been developed. This method consists of three components: (1) a laser, (2) a photosensitizer, and (3) an immunoadjuvant. The laser used was an 805 nm diode laser which can penetrate organized tissue with little energy deposition. The photosensitizer, indocyanine green (ICG), is a non-toxic dye with an absorption peak around 790 nm. It has been used in lesion detection [9–11], in enhanced laser treatment [12,13], and used for hepatic, biliary, cardiovascular and ophthalmic studies in humans [14–19]. A glycated chitosan gel (GCG) was used as the immunoadjuvant; chitosan has high biodegradability and low toxicity [20,21] and it has been shown to be an immunostimulus [22–27]. In addition, GCG also functions as a carrier of ICG and prolongs the retention of the dye at the injection site. We applied this method to treat a chemically induced, transplantable, metastatic rat mammary tumor (strain DMBA-4 [28–30]) and recorded both the immediate response and the long-term impact of the treatment.

2. Materials and methods

2.1. Animals

Wistar Furth female rats (Harlan Sprague–Dawley, Inc., Indianapolis, IN), aged 6–7 weeks and weighing 100–150 g, were fed with Custom High Polyunsaturated Fat Diet (ICN Biomedicals, Aurora, Ohio) throughout the experiment to facilitate tumor growth. After laser treatment, the rats were housed in individual cages. A total of 148 rats were used in our study; 33 were tumor-bearing control rats and rest were treated by laser at various power and duration settings, in conjunction with different dosages of photosensitizer and immunoadjuvant.

2.2. Tumor transplantation

The cancer cells for the animal model used in our experiments were derived from the chemically induced, transplantable, rat mammary tumor strain DMBA-4 [28–30]. Approximately 250 000 live cells were injected subcutaneously into one or both inguinal fat pads of female Wistar Furth rats and the animals were monitored daily for tumor development.

2.3. Photosensitizer and immunoadjuvant administration

Indocyanine green (Sigma Chemical Co., St. Louis, MO) was purchased in dry powder form. Glycated chitosan gel was prepared by incubating an aqueous suspension of chitosan with a three-fold excess of galactose and subsequent stabilization by borohydride reduction of the Schiff bases. The ICG-chitosan solution was prepared by grinding in an all glass homogenizer a known weight of the dry ICG powder in sufficient glycated chitosan gel to yield a solution of desired final concentration. Volumes of 70–500 μl of 0.25–1% ICG solution in 1% glycated chitosan gel were injected into the center of tumors prior to the laser treatment.

2.4. Laser treatment of tumor

The Diomed 25 (Diomedics, The Woodlands, TX), a diode laser of 805 nm wavelength, was used in all experiments. Several experimental protocols with laser parameters of 2–15 W and exposure duration from 3 to 10 min were investigated. The laser energy was delivered through an optical fiber (1.2 mm in diameter) to the treatment site in a non-contact mode. The fiber tip was maintained at a distance of 4 mm from the skin surface and was slowly moved across the entire tumor.

2.5. Post-treatment observation

Post-treatment examination was made daily and the dimensions of the tumors were measured twice

weekly. The volume of tumors was calculated, assuming an ellipsoid, using $V = 4/3\pi abc$ where a is the semi-major axis, and b and c are the semi-minor axes. The average density of the tumor, measured as 1.05 g/cm^3, was utilized to calculate tumor burden.

3. Results

3.1. Effect of tumor treatment

The experimental rats, depending on the post-treatment course, fell into three discrete groups: (a) unresponsive (i.e. death at around 30 days, same as control tumor-bearing rats [8]); (b) positive response (rats survived up to 45 days, a 50% increase of the expected life span); and (c) success (tumor eradication and long-term survival up to 90 days post tumor implantation). The treatment yielded an average of 14% positive response and 8% success (see Table 1). The response to the treatment was clearly affected by both the level of laser energy and the length of laser exposure. It was evident from our experimental results that the treatment with lower laser powers (below 5 W) and longer exposure duration (above 2 min) was more effective. The two most recent experimental groups (16 rats), when treated with 2 W for 10 min using 200 μl 0.25% ICG in 1% GCG, yielded 50% positive responses and 25% long-term survivals. One experimental group (six rats) even yielded a 50% success rate. The treatment of early stage tumors also appeared to be most effective, since the photothermal destruction of smaller tumors tended to be more complete. Likewise, earlier initiation of the immune response may prevent metastatic seeding to remote sites.

3.2. Positive impacts: short- and long-term

The immediate effect of the laser-photosensitizer treatment was the destruction of tumor cells due to the photothermal interaction of the laser and ICG. All the rats with positive responses had smaller tumor burdens, about half the volume of the control tumor-bearing rats, at the time of death. Among all the long-survival rats, the tumor profile, both treated primary tumor and untreated metastasis, was unique: after treatment, the tumor growth continued but at a slower rate, and at a certain point (range 4–6 weeks), the mass began a gradual reduction. Fig. 1A–D shows the onset and the magnitude of such responses, using tumor profiles of three treated rats with and without metastases.

3.3. Tumor re-challenge

Five tumor-bearing rats, cured following laser-ICG-GCG treatment, were rechallenged with three times the standard dose of tumor cells, and no tumors developed. Sixty days after the first rechallenge, three of the rats were challenged again with the increased dose of tumor cells but the rats still remained refractory to the rechallenge. Meanwhile, 12 untreated rats of the same age all died within 30 days of tumor transplantation.

4. Discussion

The DMBA-4 tumor cell line used in these experiments is an aggressive strain in female Wistar Furth rats; 99% of tumor-bearing rats died around 30 days after tumor cells were implanted, even with effective tumor cell killing through photosensitizer-enhanced laser treatment [8]. Chemotherapy has been shown only to slow tumor metastases in this tumor model, but neither positive response nor long-term survival could be achieved (unpublished data). The results obtained (Table 1 and Fig. 1) indicated that an immunological reaction played a major role in the success of our treatment. Subsequent studies showed that our protocol triggered a humoral immune response. This deduction is based on the following observations: (1) the full scale reduction of tumor burden started approximately 4 weeks after the treatment, as demonstrated in Fig. 1; (2) at this point in time there was no evidence of increased lymphocyte or macrophage presence in histological preparations; (3) the resistance to tumor rechallenge induced by the treatment; and (4) the lack of tumor-specific, cell-mediated immunity observed in the study of cell-dependent cytotoxicity using ^{51}Cr labeling technique (unpublished data). Our preliminary immunohistological results (unpublished data) also demonstrated that serum from successfully treated tumor-bearing rats contains antibodies bound strongly to tumor cells, both live and preserved. Our

laser-ICG-GCG treatment appeared to induce and enhance a stronger immune response that led to total eradication of primary and metastatic tumors and to a long-lasting resistance to subsequent tumor challenge. Because of the short life-span of the tumor-bearing rats (approximately 30 days), the humoral response often may not be established early enough to effectively combat the tumor cell proliferation and metastases. Less aggressive animal tumor models, and particularly tumors in humans, should be more sus-

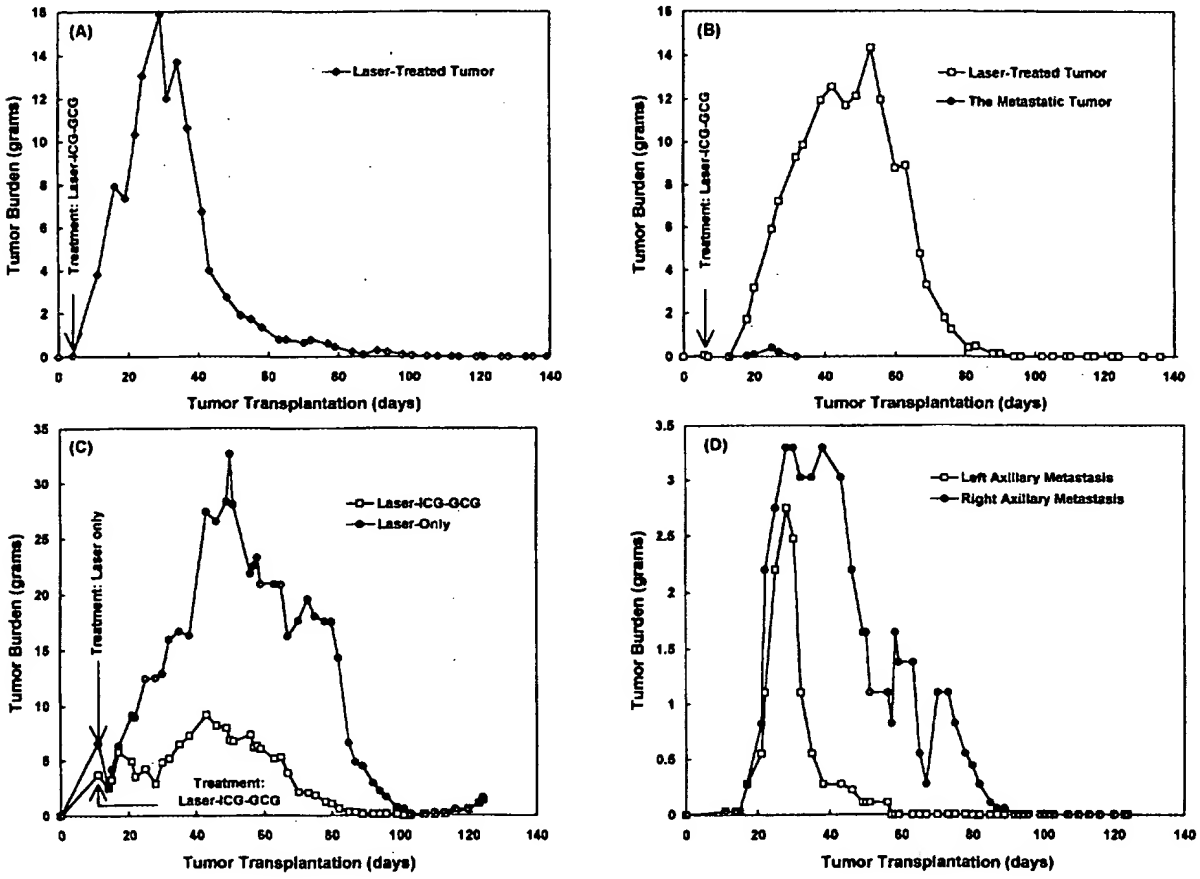

Fig. 1. The tumor burden profiles of laser-ICG-GCG-treated long-term survival rats (>90 days). (A) Rat without metastasis. The tumor cells were inoculated only in the right inguinal fat pad. A single laser treatment (arrow), 2 W for 10 min, was applied after intratumor administration of ICG-GCG solution (200 μl of a 0.25% solution of ICG in 1% glycated chitosan injected 4 h prior to laser treatment). The primary tumor continued to grow, reaching its maximum volume around 35 days after treatment and then began to recede. The tumor completely disappeared around day 100. No tumor recurrence was observed. (B) Rat with a short-lived metastasis. The tumor cells were inoculated only in the left inguinal fat pad and a single laser treatment of 2 W for 10 min (arrow) was applied. Two hundred μl of a 0.25% solution of ICG in 1% glycated chitosan was injected 1.5 h prior to laser treatment. The treated primary tumor (open squares) underwent a course similar to that shown in (A), reaching its maximum 47 days after the treatment and disappearing around day 90. The small tumor metastasis to the right inguinal area was observed around day 18 and it disappeared in less than 2 weeks (solid circles). No tumor recurrence was observed. (C,D) Long-term surviving rat with metastases and recurrence. The tumor cells were inoculated to both left and right inguinal fat pads before the treatment. The left primary tumor was treated with laser-ICG-GCG and the right tumor with laser only. The laser parameters were 5 W and 3 min. Seventy μl of 1% ICG in 1% glycated chitosan was injected into the left inguinal tumor 24 h prior to laser treatment. The primary tumors (C) underwent courses similar to that shown in (A) and (B); the laser-ICG-GCG treated tumor (open squares) grew at a much slower rate than the laser-only tumor (solid circles). The metastases to both left and right axillary areas became noticeable around day 15, peaked between day 30 and 40, and then disappeared gradually as shown in (D). Tumor recurrence was observed at the primary sites but the rat died while the recurrent tumor load was relatively small, 124 days after tumor transplantation.

Table 1
Effect of the laser-ICG-GCG treatment on tumor-bearing rats

Group (no. of rats)	Treatment time (days after inoculation[a])	ICG + chitosan administration[b]	Laser treatment[c]: power and duration	Positive response[d] number (%)	Long-term survival[e] number (%)
1 (37)	10–15	1% ICG; 1% GCG; 70–100 μl	5 W; 3–6 min	3 (8)	3 (8)
2 (3)	16	1% ICG; 1% GCG; 150 μl	15 W; 3 min	0 (0)	0 (0)
3 (27)	10–15	0.5% ICG; 1% GCG; 70–400 μl	3–5 W; 3–10 min	2 (7)	2 (7)
4 (13)	10–15	0.25% ICG; 1% GCG; 100–400 μl	5 W; 5 min	1 (8)	0 (0)
5 (19)	7–8	0.5% ICG; 1% GCG; 100–500 μl	3–5 W; 3–10 min	2 (11)	0 (0)
6 (10)	4	0.25% ICG; 0.5% GCG; 200 μl	2 W; 10 min	4 (40)	1 (10)
7 (6)	6	0.25% ICG; 0.5% GCG; 200 μl	2 W; 10 min	4 (67)	3 (50)
8 (33)	No treatment[f]	–	–	0 (0)	0 (0)

[a]Tumor transplantation: 250 000 cells injected into the inguinal fat pad, in most cases either left or right and in some cases both.
[b]The ICG solution in glycated chitosan gel was injected directly into the center of the tumor between 0 h and 24 h prior to laser exposure, in most cases to either left or right inguinal tumor and in some cases both.
[c]The energy of an 805 nm solid state laser was directed to the treatment sites through a 1.2 mm fiber which remained in a non-contact mode (4 mm distance from the skin surface); in most cases either left or right and in some cases both tumors were treated.
[d]The positive response is defined as the survival time longer than 45 days after tumor transplantation, which is a 50% increase in survival. This group also included the rats that continued on to be long-term survivors.
[e]The long-term survival is defined as the survival time longer than 90 days after tumor transplantation.
[f]Survival time (± SD) 31.5 ± 3.7 days.

ceptible to this treatment, since they would allow this laser-sensitizer-assisted immunotherapy to achieve a maximum response before the hosts reach the moribund stage.

In summary, our treatment protocol constitutes a novel approach among laser-based modalities for the treatment of malignant tumors. We have shown that the sensitizer-enhanced photothermal interaction, as well as possible photochemical interactions as yet to be specified, destroys targeted tumor cells on a large scale and in a circumscribed fashion. The glycated chitosan then elicits an immune reaction against the remaining population of tumor cells, by combining with cellular antigens released from disrupted tumor cells to form an in situ autovaccine. This hypothesized tandem effect is consistent with the local and systemic response observed after laser-ICG-GCG treatment. We suggest that the successful eradication of the tumors and subsequent resistance to tumor challenge were the result of a significant response by the immune system, primarily the humoral arm, as evidenced by our post-treatment observations. It is possible that other lasers, immunomodulators and sensitizers could be employed using the same principles. Further investigation is currently in progress involving large groups of animals and applying different treatment parameters in order to determine the efficacy of our treatment modality. Immunological studies are also in progress in order to understand the working mechanism of this novel method.

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Evaluation of serum levels of tumour necrosis factor-alpha (TNF-α) and soluble IL-2 receptor (sIL-2R) and CD4, CD8 and natural killer (NK) populations during infrared pulsed laser device (IPLD) treatment

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SUMMARY

The purpose of this study was to evaluate serum levels of TNF-α, sIL-2R and distribution of peripheral leucocyte subsets in patients with advanced neoplastic disease undergoing IPLD treatment. Fifteen cancer patients with evidence of persistent disease were further divided in two groups according to outcome at the end of the period of clinical evaluation: group 1 patients were still alive and group 2 patients had died. Our results show: (i) an increase in the initial level of TNF-α in both groups; (ii) a decrease in TNF-α levels during the follow up of group 1 patients; (iii) a significant increase in serum levels of sIL-2R in patients in group 2 compared with those in group 1; (iv) a progressive and constant increase in TNF-α levels in group 2; (v) a decrease in $CD4^+CD45RA^+$ subpopulation in both groups; (vi) an increase in $CD25^+$ cells; (vii) an increase in $CD4^+$, $CD4^+CD45RA^+$ and $CD25^+$ cells during the follow up of group 2 patients. The data generated here form the basis for further investigations on the use of IPLD as a single agent and in combination with other biological response modifiers in cancer patients.

Keywords cancer laser cytokines tumour necrosis factor-alpha sIL-2R cytofluorometry

INTRODUCTION

Neoplastic diseases cause a sustained and complex alteration of the immune system, including cytokine secretion. Cytokines are a group of polypeptides which play a major role in the modulation of host immune responses, and possibly also in immunopathologic mechanisms [1].

For example, studies with recombinant TNF-α have revealed numerous cell regulating activities of this cytokine, indicating that it is a very important mediator of inflammation and immunity. TNF is cytotoxic/cytostatic for several tumour cell lines *in vitro* [2–4], but can also be cytotoxic for normal cells under certain *in vitro* conditions [5]. Moreover, the majority of tumour cell lines are TNF-resistant [3], and there is evidence that endogenous production of TNF by tumours may be linked to their growth mestastasis, while the neutralization of TNF activity may be of potential therapeutic benefit [6].

IL-2 is an essential cytokine which plays a central role in the immune response [7] and is released after activation of T lymphocytes. In addition to the expression of IL-2 receptors on the cell surface, a soluble form of the IL-2 receptor (sIL-2R) can be released in the serum [8]. Abnormally high levels of sIL-2R have been described in different diseases [9], and it has been proposed as a marker of the host response in patients with neoplasms.

Several studies have also defined functionally distinct subsets of human lymphocytes that display a variety of regulatory and effector functions [10]. The major division of these subsets is between cell populations which bear CD4 and CD8 antigens (helper or cytotoxic/suppressor cells respectively), but also significant are CD16 and CD56 markers (natural killer cells) and CD19 antigen (mature B cells) [10]. The quantification of these subpopulations in cancer patients is an important parameter for evaluating responses induced by the neoplasm.

The bioeffects of laser radiation have been widely reported in the medical literature [11,12]. These are generally manifested as biochemical, physiological or proliferative phenomena. The cell type most widely used in these studies has been the lymphocyte, in which laser radiation induces changes at the level of DNA and RNA synthesis, expressed by acceleration of proliferation and, consequently, stimulation of the cellular immune response [13].

The present study was designed, first, to undertake a sequential examination of immunological parameters (serum levels of TNF-α and sIL-2R, fluorocytometric evaluation of peripheral lymphocytes) in patients with advanced neoplastic

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Table 1. Diagnosis, stage, previous therapeutic experience and evolution in patients with advanced tumours treated with IPLD ($n=15$)

Diagnosis	TNM	Previous treatment	IPLD treatment (months)	Outcome
ADC colon	IV	SUR, RT, CHT	19	Alive
ADC colon	IV	SUR, CHT	10	Alive
Malignant meningioma	IV	SUR, RT, CHT	33	Alive
Chondrosarcoma	III	SUR, RT	17	Alive
Osteoblastoma		—	SUR	17 alive
ADC parotid	IV	SUR, RT	22	Alive
Transitional meningioma	II	SUR	14	Alive
ADC gallbladder		IV	SUR	12 deceased
ADC oesophageal	IV	SUR	7	Deceased
VIPoma	IV	SUR, CHT	4	Deceased
Breast cancer	IV	SUR, RT, CHT	12	Deceased
Breast cancer	IV	SUR, RT, CHT	8	Deceased
Breast cancer	IV	SUR, RT, CHT	6	Deceased
ADC colon	IV	SUR, RT, CHT	8	Deceased
Cancer of the lung	IV	RT	13	Deceased

ADC, Adenocarcinomas; SUR, surgery; CHT, chemotherapy; RT, radiotherapy.

disease, undergoing IPLD treatment, and second, to demonstrate a possible correlation between pretreatment immunological values and the response to IPLD.

PATIENTS AND METHODS

Study subjects

A pilot study included 15 patients with histologically confirmed cancer refractory to conventional therapy, eight females and seven males with an age range of 25–59 years. Table 1 shows the diagnosis, TNM stage, previous therapeutic experience, time of treatment and evolution of the patients after IPLD. Group 1 ($n=7$) consisted of five neoplasias with metastatic disease (two adenocarcinomas (ADC) of the colon, one malignant meningioma, one ADC of the parotid), two neoplasias with a low degree of malignity (one chondrosarcoma of the posterior cranial space and one transitional meningioma), and one benign tumour (one osteoblastoma of the cervical column). Group 2 patients ($n=8$) had neoplasias with metastatic disease (three breast cancer, one ADC of the colon, one ADC of the gallbladder, one ADC of the oesophagous, one cancer of the lung, one VIPoma). All patients had been previously treated by surgery, chemotherapy and/or radiotherapy, either combined or alone. When they were accepted into the protocol, all had active and progressive disease, with a mean of 8 months (range 6–14 months) since their last treatment by radio and/or chemotherapy. For those who had undergone surgery, this had taken place a mean of at least 10 months (range 2–24 months) before the beginning of IPLD treatment. All requirements of the Helsinki protocol were fulfilled. The criteria of selection included: a Karnowsky status of >40, a life expectancy greater than 3 months, no mental disturbance, no steroid treatment, no depressive state or inadequate family cooperation. The patients were divided into two groups according to their status at the end of the period of clinical evaluation: group 1 patients were still alive, while group 2 patients had died during the course of the protocol. Clinical studies of the effects of IPLD were commenced before the initiation of immunological evaluation in all of the patients, and thus pre-therapy values were not available for them. Patients were evaluated several times during the course of the protocol. We arbitrarily grouped results according to the period of time of treatment: initial evaluation (before IPLD treatment when available), 1–4 months, 5–12 months, 12–30 months and >30 months. Informed consent was obtained from patients before entry in the study, in accordance with individual institutional policies.

A group of 45 healthy donors were also evaluated; these included 29 women and 18 men with an age range of 25–50 years.

Therapeutic laser apparatus

The therapeutic laser apparatus [14] has been designed for treating neoplasias and other systemic diseases through the delivery of infrared laser radiation via the biological circuitry of the patient. Briefly, the pulse train is selected at between 0·5 MHz and 7·5 MHz, with a relatively low duty cycle to avoid thermal energy from making the process uncomfortable. The laser beam is applied perpendicularly to the surface of the patient's skin in close proximity to the vascular interstitial closed circuit (VICC) (biological circuit which most efficiently carries laser energy to the target tissue). This procedure is repeated periodically and the clinical response monitored through conventional nuclear magnetic resonance images or other accepted methods.

Cytokine assays

sIL-2R and TNF-α were measured by an ELISA technique. Peripheral blood from patients and controls was collected by venepuncture without anti-coagulant and left to coagulate for 1 h at room temperature. The serum was collected and kept frozen at −80°C until assay. Although there is some controversy concerning the measurements of cytokines on sera, and since several proteases can be released during clotting events, we decided to use sera because most previous work has been done

Table 2. Levels of TNF-α (pg/ml) in sera of patients with advanced cancer during treatment with IPLD

Group	Patients (*n*)	Months 0–1	2–4	5–12	12–30	>30
1	7	33·2±15·1† (6·0–74·0)*	49·1±13·1‡ (6·0–122·0)	145·6±26·2‡ (6·0–400·0)	102·3±16·4‡ (6·0–300·0)	35·0±5·0
		NS§	NS	NS	NS	
2	8	16·4±2·6¶ (6·0–25·0)	81·5±211·4‡ (6·0–320·0)	166·9±27·2‡ (13·0–320·0)		
Controls	47	11·5±2·5‡ (6·0–40·0)				

* Range of values.

† Statistically significant differences ($P<0·05$) in patients from group 1 according to period of evaluation: 0–1 month *versus* 5–12 months and period 2–4 months *versus* 5–12 months and 12–30 months.

‡ Statistically significant differences ($P<0·05$) between patients in groups 1 and 2 according to period of treatment *versus* controls.

§ Comparison by Student's *t*-test between groups 1 and 2. NS, Not significant.

¶ Statistically significant differences ($P<0·05$) in patients from group 2 according to period of evaluation: 0–1 month *versus* 5–12 months and period 2–4 months *versus* 5–12 months.

on sera. The TNF-α kit was provided by Genzyme (Boston, MA) and sIL-2R by Immunotech A.S. (Marseille, France). For TNF-α evaluation, monoclonal anti-TNF-α was diluted in coating buffer and 100 μl added to each well of 96-immunoplate wells. After overnight incubation at 4°C, the plate was washed three times with washing buffer. Serum samples and appropriate negative and positive controls were added in duplicate wells and incubated for 2 h at 37°C. After four washings, polyclonal rabbit anti-TNF-α was added to each well of a 96-plate well (1 h, 37°C). Alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulin was dispensed into each well and incubated for 1 h at 37°C. After four washings, substrate reagent was added and after incubation at room temperature, the plate was read on a standard ELISA reader at 405 nm. For sIL-2R measurements, plates precoated with rat anti-IL-2R MoAb (11H2) were washed four times with washing buffer and incubated with standard or serum samples (40 μl) and a rat anti-IL-2R MoAb conjugated to peroxidase (33B3, which recognizes an epitope distinct from that recognized by 11H2) for 2 h at 37°C with shaking. After four more washings, plates were incubated for 30 min with substrate buffer and read at 490 nm on a standard ELISA reader. Cytokines were considered as detectable when TNF-α was >12 pg/ml and sIL-2R >70 pM/ml. When patients were studied several times, individual serum samples were always included on the same plate to avoid variation between plates.

Cytofluorometric analysis

Peripheral blood (100 μl) was incubated with 5 μl of different MoAbs coupled to either FITC, rhodamine (RD-1) or PE. Those employed for all patients were CD4 (T4), CD8 (T8), coupled to RD-1, CD3 (T3), CD25, 4B4 (CD29), 2H4 (CD45RA), coupled to FITC (Coultronics, Hialeah, FL) and Leu-11c (CD16) coupled to PE (Becton Dickinson, MA). After 30 min of incubation at room temperature, the blood cells were washed twice and the erythrocytes lysed (Immunolyse, Coultronics). Cell counts were performed in an 'EPICS 753' (Coultronics) equipped with a 5 W argon dye laser, and fluorescence was displayed on a logarithmic scale. For two-colour analysis, a 530 nm short-pass filter for FITC and 590 nm long-pass filter for PE were employed. Green (FITC) and red (PE) fluorescence were measured on lymphocytes gated at forward and right-angle scatters. Results were expressed as per cent of total lymphocytes.

Statistical analysis

The statistical significance of the differences between the mean±s.d. of the values of the two groups of patients and between the different periods of evaluation within a group was estimated by the normal or paired Student's *t*-test respectively. The Spearman rank correlation coefficient was used to determine the significance of the different correlations calculated. The proportions of patients with positive results for the different evaluations were compared by the χ^2-test.

RESULTS

Serum levels of cytokines in normal controls and cancer patients

The serum levels of TNF (Table 2) at the beginning of IPLD treatment were low and not statistically different between the two groups of patients and the normal controls. However, 60% of patients in group 1 and 83% in group 2 had detectable TNF-α levels (>12 pg/ml), compared with 21% in the control group. The difference between group 2 and the controls was statistically significant ($P<0·005$).

A significant increase ($P<0·05$) in TNF-α was found in group 1 after 5–12 months of treatment, compared with both their initial values and values at 2–4 months of treatment. This difference was still observed at 12–30 months of treatment. However, upon the last evaluation of two patients from this

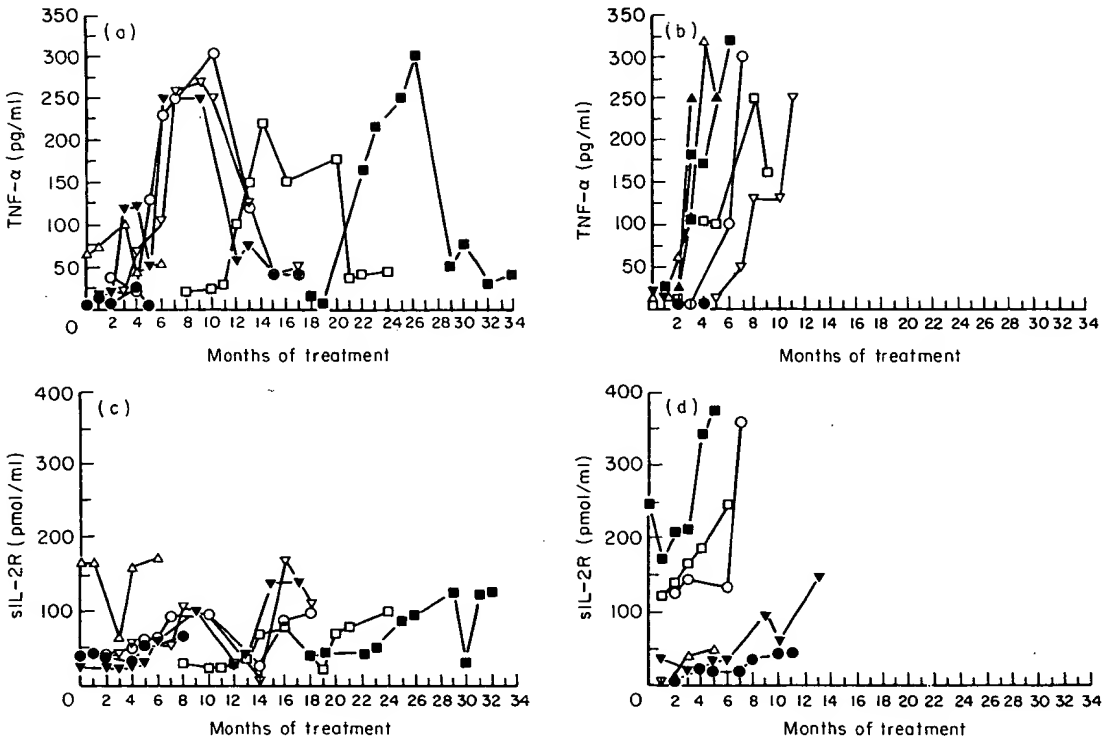

Fig. 1. Evolution of TNF-α and sIL-2R levels during IPLD treatment. Patients were divided in two groups according to outcome: survivors ($n = 7$, (a), (c)) and patients who died ($n = 7$, (b), (d)). For each patient evolution of serum levels is shown. The months of IPLD treatment are indicated. (a) TNF in surviving patients. (b) TNF in patients who died. (c) sIL-2R in surviving patients. (d) sIL-2R in patients who died.

Table 3. Levels of sIL-2R (pM) in sera of patients with advanced cancer during treatment with IPLD

Group	Patients (n)	Months 0–1	2–4	5–12	12–30
1	7	87·9 ± 31·7† (24·6–165·0)*	49·3 ± 11·7 (23·7–160·0)	66·9 ± 8·3 (23·9–171·0)	66·9 ± 8·3 (6·0–166·0)
		NS‡	$P < 0.05$	NS	
2	8	116·2 ± 43·8†§ (6·0–246·0)	117·8 ± 24·6 (6·0–340·0)	118·2 ± 32·7 (19·0–375·0)	
Controls	47	37·2 ± 4·2§ (6·0–40·0)			

* Range of values.

† No statistically significant differences ($P > 0.05$) in group 2 patients at the different periods of treatment.

‡ Comparison by Student's t-test between groups 1 and 2. NS, Not significant.

§ Statistically significant differences between group 2 patients and controls at initial evaluation.

group who had more than 30 months of treatment, values had decreased to normal.

We also observed a significant increase ($P < 0.05$) in TNF-α values in group 2 after 2–4 months of treatment; this increase continued to the time of their deaths, when the values were significantly higher ($P < 0.05$) compared with earlier times of treatment.

Individual results of the different evaluations performed in patients from the two groups are shown in Fig. 1. In patients from group 2, we observed a progressive and constant increase in TNF-α up to the time of death. In contrast, in patients from group 1, the levels of TNF-α increased steadily from their initial evaluation, but then decreased during the course of treatment.

In contrast to TNF values, the evaluation of sIL-2R (Table 3) demonstrated high levels of this marker upon initial evaluation of both groups of patients when compared with controls, although this difference was statistically significant only for group 2. Moreover, the percentage of patients with significant

Table 4. Comparison of surface markers in cancer patients and controls

Cell subset	Patients ($n=15$)	P	Controls ($n=11$)
CD4	34·14 ± 8·1	< 0·01	42·24 ± 6·0
CD8	21·77 ± 7·7	NS	22·5 ± 5·9
CD3	68·36 ± 12·4	NS	62·67 ± 9·8
CD25	6·17 ± 6·36	< 0·01	1·94 ± 1·22
CD16	8·10 ± 7·6	NS	6·51 ± 4·9
$CD4^+CD29^+$	24·67 ± 10·0	NS	24·28 ± 7·7
$CD4^+CD45RA^+$	12·7 ± 6·4	< 0·05	18·79 ± 9·7

Results are expressed as mean ± s.d. per cent of positive cells among total lymphocytes.

values of sIL-2R (> 70 pM) was higher in group 1 (40%) and group 2 (60%) when compared with controls (0%), with this difference also statistically significant between group 2 and controls. When we compared the sIL-2R levels between patients from group 1 and group 2 at different periods of evaluation, the general tendency showed higher values for group 2 than for group 1 at all times, but this difference was statistically significant ($P < 0·05$) only at 2–4 months of treatment.

During treatment (Fig. 1) we observed an increase in serum values of sIL-2R in the second group of patients, while group 1 had only minor variations. An analysis of the Spearman correlation coefficient between TNF and sIL-2R for patients from both groups demonstrated a positive and significant ($r = 0·436$; $P = 0·008$) correlation for patients from group 2, but not for patients from group 1 ($r = 0·09$; $P = 0·47$).

Cytofluorometric analysis of lymphocyte subsets

Table 4 shows the percentages of different lymphocyte subsets in controls and in cancer patients from both groups when studied at the beginning of the protocol. We found a significant decrease in $CD4^+$ and in $CD4^+CD45RA^+$ in cancer patients when compared with normal controls ($P < 0·01$). In contrast, $CD25^+$ cells were significantly increased in the patients ($P < 0·05$). No significant differences were found in CD8, CD3, CD16 and $CD4^+CD29^+$ populations between patients and controls.

The lymphocyte subsets were followed in five patients from group 1 during IPLD treatment (results not shown). A tendency toward an increase occurred in all five patients evaluated for $CD4^+$, and in three patients who were evaluated for $CD4^+CD45RA^+$ and $CD25^+$. No consistent changes were found for CD8, and in all five patients a slight decrease in $CD16^+$ cells was observed.

An analysis of the Spearman correlation coefficient shows a positive and highly significant ($r = 0·769$; $P = 0·009$) correlation between the serum levels of sIL-2R and the expression of CD25 marker at the surface of lymphoid cells, measured by fluorocytometry, in patients from group 1, but not in patients from group 2.

DISCUSSION

This study was designed to determine immunological effects in patients with advanced neoplastic disease undergoing IPLD treatment. Neoplastic disease is known to have a profound influence upon immune responses. Growing tumours have been associated with decreasing immunocompetence, including disturbances in cells concerned with immunoregulation [15].

In the present report, we evaluated TNF-α and sIL-2R in the sera of cancer patients treated by IPLD. It should be noted that controversy exists concerning the validity of both biological and immunological methods for the detection of cytokines. The advantage of biological methods lies in the fact that, by definition, they measure the biological activity of the studied cytokine. However, it cannot be excluded that other cytokines also present in the biological sample may influence the results. Immunological methods specifically measure the cytokine in question, but do not provide information about its biological activity. Given the wide application and acceptance of the immunological methods, we chose to employ ELISA in the present study.

The role of TNF during immunological responses against tumour cells is still unclear. Early production of TNF could select TNF-resistant tumour cells, and TNF might be secreted by cancer cells themselves [16,17]. Moreover, late production of TNF has been associated with poor outcome for patients, deleterious inflammation-related phenomena and poor response to treatment [6]. This is in agreement with our results, since we demonstrated that the levels of TNF increased progressively in patients from group 2 until the moment of death. In contrast, the decrease in TNF levels observed in patients from group 1 was associated with a better clinical course for these patients. These results are consistent with the hypothesis that an inactive and necrotic tumour that is regressing is not able to produce TNF or to stimulate TNF production by other cells. Similar results have been obtained in patients with malignancies in which complete remission has been achieved [6].

This argues in favour of the possibility that IPLD can play a role in reestablishing regulatory mechanisms of the immune response that were altered by growing tumours. We speculate, therefore, that serum levels of TNF may be of potential value in the follow up of cancer patients during IPLD treatment and that a decrease in TNF-α could be indicative of a better clinical response.

We also studied the serum levels of sIL-2R. IL-2, which is a pivotal cytokine in T cell differentiation and activation, acts via an interaction with high affinity cell membrane receptors (IL-2R). The high affinity receptor consists of two chains of 75 kD and 55 kD. The CD25 antibody detects 55-kD sIL-2R which is a circulatory form of the 55-kD chain. CD25 has been reported to be increased in several clinical situations, with an imbalance of immune homeostasis [18–20].

Our results showed that serum levels of sIL-2R were significantly increased in three of these patients in group 2 compared with those in group 1. Moreover, the level of sIL-2R was significantly higher at initial evaluation in most patients in group 2, and this tended to increase even more in four of the six patients before their deaths. The mechanism responsible for the increased release of sIL-2R in patients with disseminated solid tumours is still obscure. However, it could be due to unknown factors produced by cancer cells themselves, capable of affecting the normal expression of IL-2R at the cell surface [21]. It has been suggested that high levels of sIL-2R may contribute to the poor prognosis of cancer patients by blocking IL-2-mediated cytotoxic activities, since sIL-2R is capable of binding to free IL-2 [22].

Similarly, various authors have reported that a favourable response to treatment in patients with advanced cancer is associated with a reduction in the serum levels of sIL-2R [20]. In our patients, levels of this marker were somewhat high during the course of IPLD treatment in patients from group 1. This might reflect a cellular immune process in the region of the tumour or activation of the immune system. Indeed, we found a significant and strongly positive correlation between the serum level of this receptor and $CD25^+$ cells in patients from group 1. It has been well documented that expression of this receptor by cells of the lymphoid system (CD4 and CD8) is a sign of cellular activation [10]. Thus, this correlation suggests the possibility that the soluble receptor measured in the serum could be, in part, produced by activated T cells, and could thus reflect the immune response of the host against its tumour. The production and regulation of IL-2 and its receptor are closely related. Sharma *et al.* [23] were unable to correlate sIL-2R levels with CD25 expression on lymphocytes in women with breast cancer, but they found a significant negative correlation between this marker and lymphocytes within the tumours in this same group of patients. This indicates that sIL-2R exerts an immunomodulatory effect on blood lymphocytes by preventing their infiltration into the tumour tissue.

Our results suggest that high levels of sIL-2R at the beginning of the treatment could have a prognostic value in determining the response of the patients to IPLD. It is possible that treatment with IPLD produces variations in the host–tumour relationship, due to a decrease in tumour load in those patients who reacted positively to it. In contrast, the exaggerated production of this receptor could be a reflection of an imbalance in T cell subset homeostasis, in such a way that treatment with IPLD is unable to control it.

Expression of surface markers has revealed differences between advanced cancer patients at the beginning of evaluation, and controls in percentages of $CD4^+$,$CD4^+$ CD45RA and $CD25^+$ cells. Various authors have reported a decrease in CD4 cells in progressive and metastatic cancer patients [21,24]. Our results support the concept that the decrease in CD4 cells is due to a decrease in $CD45RA^+$ cells which are inducers of suppression [25]. This indicates that immune dysfunction existed in our patients before they started IPLD treatment.

When we followed a small group of patients by fluorocytometry during IPLD treatment, it was possible to demonstrate an increase in $CD4^+$ and $CD4^+CD45RA^+$ cells. Our results are in agreement with others [26] who reported a significant increase in $CD4^+$ cells which reached normal levels after radiation therapy for cancer of the uterine cervix only in disease-free patients, whereas these levels remained low in those patients with active disease.

Because of the wide variability in the parameters measured, further research, including the follow up of a larger group of patients, is needed to confirm whether the differences we observed between the groups can be used to predict the response and outcome of cancer patients undergoing IPLD treatment.

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1. Proceedings of SPIE-The international society for optical engineering: ~~1999, Vol. 3601, (Laser-Tissue Interaction X), pp. 75-81~~ 1999, Vol. 3601, (Laser-Tissue Interaction X), pp. 82-88

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Photothermal and immunological reactions against metastatic tumors using laser-photosensitizer-immunoadjuvant

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ABSTRACT

Photothermal tissue interaction is the most common phenomenon when laser energy is deposited in tissue. Because of the sensitivity of cancer cells to temperature increase, photothermal reaction can be an effective mechanism of direct cancer destruction using lasers. Tumor-specific immune response is crucial in achieving systemic and long-term cures for cancers, particularly for metastatic cancers. Can photothermal interaction induce sufficient immunological reaction when the local destruction of tumor cells occurs? To achieve selective photothermal destruction, indocyanine green as a photosensitizer was directly injected into rat mammary tumors, followed by irradiation of 805 nm laser light. Although extensive photothermal tumor killing was achieved and tumor growth was slowed down immediately following the treatment, photothermal reaction alone was shown not sufficient in controlling the treated primary tumors and their metastases. When an immunoadjuvant was used with the indocyanine green, however, the same laser treatment not only could eventually eradicate the treated primary tumors but also eradicate the untreated metastases at remote sites. The tumor eradication went through a growth-regression process over a period of six to nine weeks post-treatment, indicating an induced immune response. The Western Blot analysis using the serum from a laser-immunotherapy cured rat showed that the tumor-specific antibody induced by the treatment had a long-lasting effect. Our experimental data indicated that photothermal interaction alone was not sufficient to slow and eventually reverse tumor growth. However, it can reduce the tumor burden and at the same time release tumor antigens to be recognized by the host immune system. Therefore, in conjunction with specific immunological stimulation using *in situ* immunoadjuvants, the selective thermal injury to tumors plays an important and a direct role in this laser immunotherapy.

Keywords: Photothermal reaction; 805 nm laser; Indocyanine Green; Glycated chitosan; Laser immunotherapy

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1. INTRODUCTION

Laser photothermal tissue interaction is the first reaction when laser energy is absorbed by tissue. Because tumor cells are more sensitive to elevation of temperature than normal tissue [1], the photothermal interaction can be an effective means in tumor cell killing. However, to have a long lasting curative modality, particularly in treating metastatic tumors, a host immune response is often required. Although laser photothermal interaction can cause direct tissue injury and indirectly induce host immune responses such as local inflammation and increases of macrophages, its long-term immunological effect is not certain.

To enhance the direct destruction of tumor tissue, a combination of laser and laser-absorbing dye has been used. [2-4] When the dye is injected into the tumor, the absorption of the laser can be highly concentrated, producing precisely targeted photothermal effect. In our previously studies, the choice of laser was the 805 nm diode laser for its tissue penetration ability and the choice of dye is indocyanine green (ICG) for its corresponding absorption peak around 800 nm. Although, some photochemical reaction of ICG, generating singlet oxygen upon excitation by light, has been observed, [5-6] the photothermal reaction is still dominant with high laser irradiance and appropriate ICG concentration in tumor.

Glycated Chitosan (GC), a novel immunoadjuvant, was added to the effective photothermal interaction using the 805-ICG combination and a new triple treatment modality was introduced. [7-9] The purpose of the addition is to induce a long-term tumor-specific immunological reaction, building upon the acute photothermal tumor tissue destruction. This method showed promising results in treating a metastatic mammary tumor model in rats. Furthermore, immunological studies showed an induced anti-tumor immunity. The current study focuses on the impact of photothermal effect with and without the introduction of the immunoadjuvant, as well as on the immunological mechanism of the laser immunotherapy.

2. MATERIALS AND METHODS

2.1. The Tumor Model and Animal Preparation

DMBA-4 metastatic mammary tumor model in Wistar Furth female rats (Harlan Sprague-Dawley, Indianapolis, IN) was used. The tumor line was maintained in our lab by serially passing viable tumor cells through living hosts. The tumor is highly metastatic. Primary solid tumors usually appeared in seven to ten days after inoculation and multiple metastases emerged around day fifteen. The tumor invasion and metastases eventually kill all the hosts within 35 days without treatment. The tumor-bearing rats were treated by laser-ICG combination and by laser-ICG-GC combination. For detailed animal care procedures, see Refs. 4 and 9.

2.2. Laser Treatment of Tumors

Tumor-bearing rats were treated when the primary tumors reached a size of 0.2 to 0.5 cm^3. Aqueous solution of ICG or mixture of ICG-GC was injected to the center of the tumor before the laser irradiation. The laser used is a near-infrared solid state laser of 805 nm wavelength (DIOMEDICS, Woodlands, TX), operated at 2 watts (CW) and 10 minutes. Indocyanine green is purchased from Becton-Dickinson (Cockeysville, MD) and the immunoadjuvant, glycated chitosan, is synthesized in our lab. A volume of 200 µl of ICG or ICG-GC solution (0.25% ICG and 1% GC) was injected, resulting in an ICG dose of 2.5 mg/kg and a GC dose of 10 mg/kg. For detailed treatment procedures, see Refs. 4 and 9.

2.3. Western Blot Analysis

As the sources of primary antibodies, sera were collected from naive and untreated tumor-bearing rats. Sera were also collected from a successfully treated tumor-bearing rat at different times after tumor

rechallenge, a second tumor inoculation with an increased tumor dose. Tumor protein as the target was extracted from $1.6x10^5$ viable tumor cells and was loaded into wells of a 10% SDS-polyacrylamide gel. Following the standard Western blot analysis procedures, the binding of antibodies from the rats' sera to the tumor protein was visualized on x-ray film using a chemiluminescent detection system (Amersham Life Science, Arlington Heights, IL).

3. RESULTS

3.1. Laser-ICG Photothermal Effect on Tumors

When the tumor-bearing rats were treated by the laser-ICG combination, the immediate photothermal tumor tissue destruction was the dominate reaction. The tumor burdens after treatment were reduced and the growth of the tumor was slowed for a short period. However, the treated primary tumor and its metastases continued to grow and eventually killed the hosts. The average survival time of the treated rats is 33.8±6.1 days, compared with that of 35.1±8.0 days of the control rats. Shown in Figure 1 is the survival rate of laser-ICG treated rats, in comparison with untreated control tumor-bearing rats.

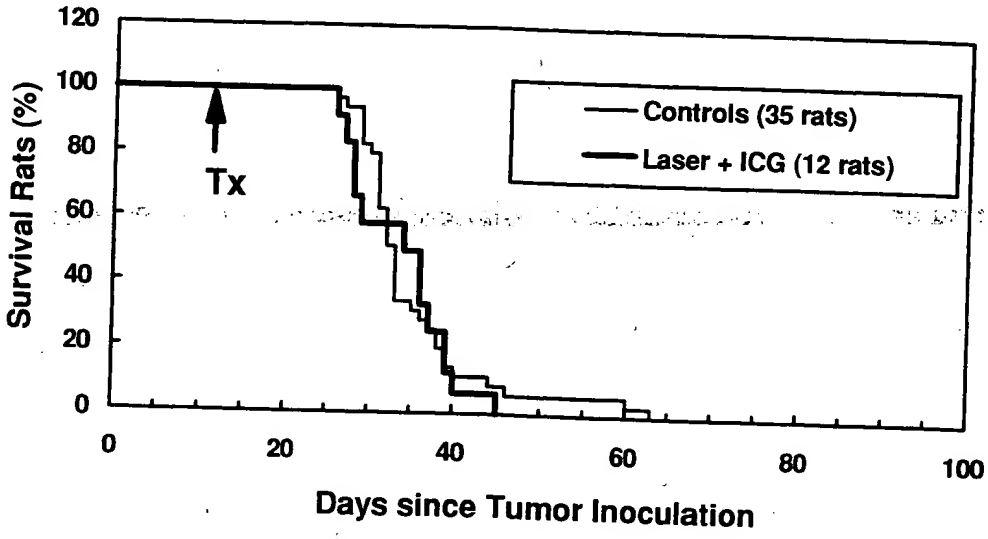

Figure 1. Survival rate of tumor-bearing rats after the treatment by laser and indocyanine green. The thin solid line represents 35 untreated control rats and the thick solid line represents 12 treated rats. The laser-ICG treatment occurred 10 days after tumor inoculation. The treatment did not affect the survival of the tumor-bearing rats.

3.2. Laser-ICG-GC Treatment of Metastatic Tumors

The addition of glycated chitosan provided a dramatic change in the outcome. Long-term survival was achieved; the average cure rate was 25% and a 40% survival rate was achieved in one experiment. Figure 2 shows the combined data from several experimental groups.

The long-term survival rats after laser-ICG-GC treatment also acquired total tumor resistance. In the subsequent tumor rechallenge with an increased tumor dosage, all the cured rats survived the rechallenge without developing tumors.

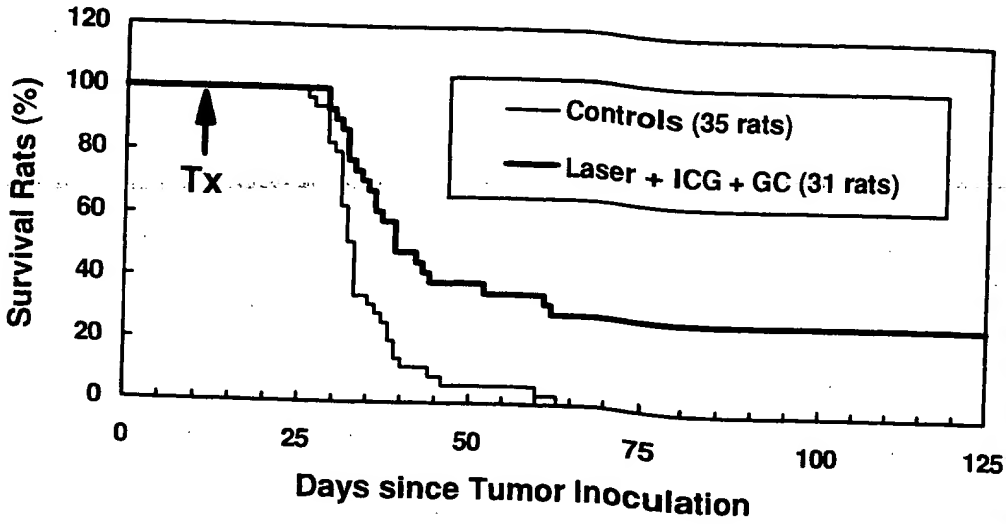

Figure 2. Survival rate of tumor-bearing rats after the treatment by the laser-ICG-GC combination. The thin solid line represents 35 untreated control rats and the thick solid line represents 31 treated rats in several experimental groups. The treatment occurred 10 days after tumor inoculation. The treatment not only significantly increased the average survival time, but also resulted in long-term survival of the tumor-bearing rats.

The laser-ICG-GC treatment also had a significant impact on the metastases. In general, 16 days after the inoculation of the primary tumor at the right inguinal area, the metastases would emerge. The first metastasis usually occurred at the axillary area on the same side of the primary tumor. Then they would emerge at the left inguinal and left axillary areas. The treatment delayed the emerging of the metastases by several days. Table I shows the average time for the metastases to emerge in one experimental group in comparison with a group of control rats.

TABLE I
Impact of Laser-ICG-GC treatment on the metastases

Metastasis	Control (20 rats)	Treated (16 rats)	Delay (days)
1st (Right Axillary)	15.7 days	19.2 days	3.5
2nd (Left Inguinal)	20.9 days	23.0 days	2.1
3rd (Left Axillary)	22.7 days	28.0 days	5.3

3.3. Tumor-Specific Antibodies Induced by Laser Immunotherapy

Figure 3 shows Western blots of tumor cell proteins probed with sera from different animals: a naive rat, a control tumor-bearing rat, and a successfully treated tumor-bearing rat. Apparently, the naive rat does not contain any tumor-selective antibody, as evidenced by the lack of staining in the Western blot (see Figure 3 a). Sera from rats successfully treated by laser immunotherapy show two distinct bands (see Figure 3 c-g) at approximately 45 kDa and 35 kDa. The 45 kDa band is also observed after probing the blot containing the tumor proteins with serum from the control tumor-bearing rat, but the intensity is much weaker (see Figure 3 b). The second band at 35 kDa is totally absent when the serum from the control tumor-bearing rat is used for probing.

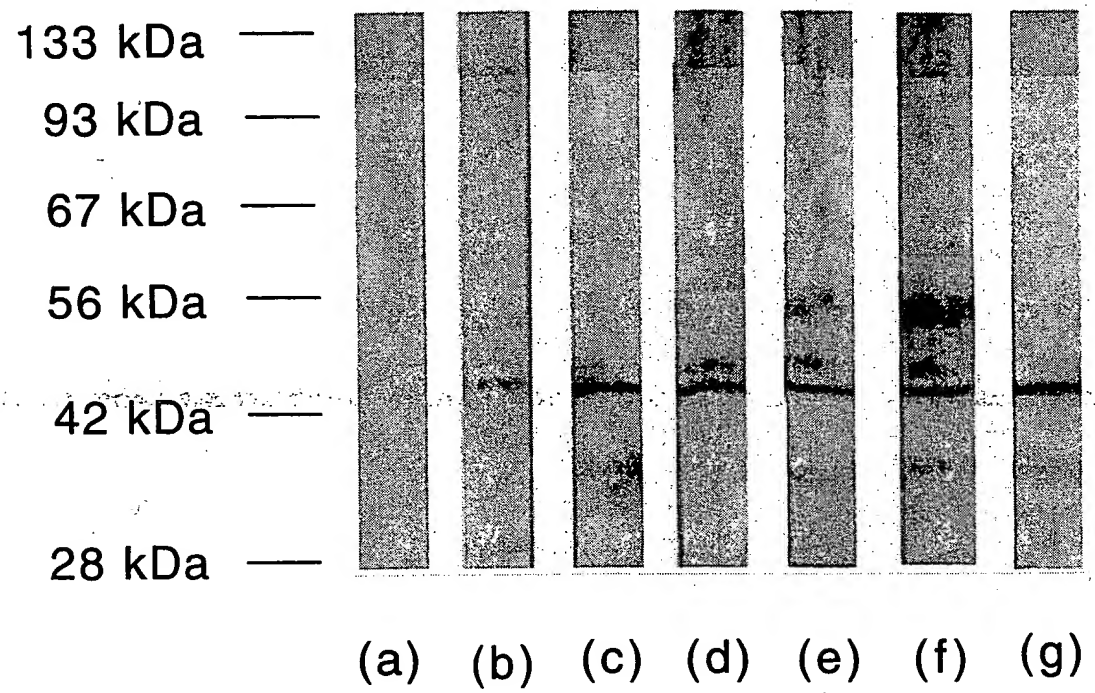

Figure 3. (a) Tumor cell proteins probed using serum of a naive rat; (b) Tumor cell proteins probed using serum from an untreated control tumor-bearing rat; (c), (d), (e), (f) and (g) Tumor cell proteins recognized using serum of a tumor-bearing rat, cured by the laser immunotherapy and 0 h (c), one week (d), two weeks (e), one month (f) and two months (g) after tumor rechallenge.

4. DISCUSSION

The photothermal interaction can be a direct and effective means of acutely eliminating tumor cells. However, our experimental results showed that photothermal interaction alone could not provide the required long-term curative effect, as shown by the laser-ICG data in Figure 1. The photothermal interaction may have induced an immune response, but certainly not one sufficient to control the tumor, especially in the case of metastatic tumors.

Laser immunotherapy resulted in a long-term and systemic effect. The 25% to 40% cure rate, as shown in Figure 2, in this aggressive tumor model indicated that this novel approach is efficacious. In addition to

the control of the treated primary tumors, the treatment had an important impact on the metastases at remote sites. The delay of the occurrence of the metastases at the different sites, as shown in Table I, indicated a systemic reaction induced by the treatment. Although the delay for the first metastasis on average was only 3.5 days, it may be crucial in the tumor control since the overall life span of the tumor-bearing rats is only about 35 days. The delay could be the time required for the host immune system to be activated and for the tumor-specific response to be developed.

The active mechanism of laser immunotherapy was apparently an induced immune response. Specifically, tumor-specific antibody which binds to the tumor protein in the region of 35 kDa and 42 kDa was found only in the sera from successfully treated rats, as shown in the Western blot analysis. More importantly, the level of the induced antibody had the same intensity over the two-month period after tumor rechallenge, indicating a long lasting immunity. However, this does not rule out an active cellular response acting in concert with the observed humoral activity.

Although certain photosensitizers used in photodynamic therapy (PDT) have been shown to be capable of inducing immune responses when activated by light.[10-14] However, their immunological effect, as a secondary function to the photochemical reaction, on cancer treatment is currently not clear. The introduction of an immunoadjuvant in our laser immunotherapy was an active measure to elicit the help of host immune system in eradicating existing tumor cells and in resisting later tumor invasion. Therefore, this triple therapy — laser, laser-absorbing dye, and immunoadjuvant — may prove to be an effective cancer treatment modality.

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1. Proceedings of SPIE-The international society for optical engineering:
   1999, Vol. 3601, (Laser-Tissue Interaction X), pp. 75-81
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12.
L22 ANSWER 104 OF 112 CANCERLIT
ACCESSION NUMBER: 86619294 CANCERLIT
DOCUMENT NUMBER: 86619294
TITLE: HEMATOPORPHYRIN DERIVATIVE PHOTOTHERAPY IN EXPERIMENTAL ONCOLOGY.
AUTHOR: Canti G; Ricci L; Franco P; Nicolin A; Andreoni A; Cubeddu R
CORPORATE SOURCE: Dept. of Pharmacology, Sch. of Medicine, Milan, Italy.
SOURCE: Non-serial, (1984) Porphyrins in Tumor Phototherapy. Andreoni A, Cubeddu R, eds. New York, Plenum Press, p. 203-12, 1984. .
DOCUMENT TYPE: (MEETING PAPER)
LANGUAGE: English
FILE SEGMENT: Institute for Cell and Developmental Biology
ENTRY MONTH: 198602
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Las rs in Surgery and Medicin 12:313–317 (1992)

Augmentation of the Systemic Host Anti-Tumor Response Through Laser Excision

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This study examines whether primary laser excision results in augmentation of the systemic host anti-tumor response to tumor rechallenge. Single R3230AC mammary tumor implants, (0.5 × 0.5 × 1.0 mm), were grown in 112 female Fisher 344 rats. The animals were randomized. Group S tumors were excised by scalpel. Group E was excised with a Surgistat electrocautery (Valley Labs, Boulder, CO). Group CS was excised with a Sharplan 1100 CO_2 laser (Sharplan, Allendale, NJ) at 25 watts (W) continuous wave (CW) (0.2 mm spot size) and the wound was "sterilized" with a 5-mm spot size by gently heating the tissue without blanching. Group K was excised with a KTP/532 laser (Laserscope, San Jose, CA) at 17 W CW using a 400 μm fiber. Group Y was excised with a Sharplan 2100 Nd:YAG laser set at 15W CW using a 0.2 mm clear sapphire tip. A second tumor implant, (0.5 × 0.5 × 1.0 mm), was placed at a remote site 14 days postoperatively. An unoperated control group was implanted. Secondary tumor volumes were measured for 36 days and the mean tumor volumes (MTV) were statistically compared. The MTV in groups CS, K, Y, and E was less than control ($P<0.01$). The MTV in groups CS, K, Y, and E was less than group S, although this was not statistically different. Lasers and cautery appear to increase the host response against subsequent tumor challenge. This study corroborates earlier studies of other modalities. Further studies to determine whether this host sensitization is an immune response and to elucidate the mechanisms of this effect are warranted.

Key words: cancer, electrocautery, experimental tumor, immunity, mammary carcinoma, surgery

INTRODUCTION

Previous studies in our laboratory [1–5] have shown that the incidence of local tumor recurrence in an experimental rat mammary tumor model is reduced when excision is performed with a laser. This effect is greatest when the tumor bed is laser-sterilized with a CO_2 laser following excision [1–5]. Possible mechanisms for this effect include thermal destruction of tumor cells, more precise tumor excision, reduced tumor seeding during surgery, alteration of the inflammatory response, and potentiation of the host immune response.

There is some evidence that other modalities can increase tumor immunity. Tumor necrosis as a result of cryosurgery, ligation, or electrcoagulation has been shown to augment tumor-specific transplantation immunity as compared to excision alone [6]. Immunity to a secondary challenge of tumor cells was greater in mice with sarcoma which were treated with electrocoagulation

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rather than limb amputation [7]. Electrocoagulation of a rabbit testicular carcinoma led to regression of tumor in the other testicle and induc d immunity to subsequent tumor challenge [8]. In a neuroblastoma model, electrocoagulation potentiated the host immune response to distant tumor as well as residual primary tumor [9]. In the same model CO_2 laser excision decreased the growth of residual tumor compared to scalpel excision, an effect attributed to increased tumor immunogenicity [10].

This study compares the growth of a secondary tumor challenge at 14 days following excision of the primary rat mammary tumor with a CO_2 laser, YAG laser, KTP laser, electrocautery, and scalpel. Fourteen days was chosen as the interval between primary excision and tumor rechallenge as two weeks is the least amount of time necessary for host sensitization to develop. In addition, this choice of 14 days serves to minimize the effect of the acute inflammatory response (due to primary excision) on the secondary tumor as the effects of surgical trauma abate after 7 days and are minimal at 14 days [11].

MATERIALS AND METHODS

One hundred twelve female, 120 g, Fisher 344 rats were implanted with R3230AC mammary tumor. Tumor pieces measuring 0.5 × 0.5 × 1.0 mm were implanted in the mammary ridge by trochar injection. Tumor growth was monitored by measuring tumor dimensions with calipers. Tumor volume was calculated as $\pi/6 \times$ length × width × depth. The rats were randomized into 5 groups of 22 each based on the tumor volume at day 14. All groups had similar mean tumor volumes and standard deviations.

Surgical excision was performed on day 21 following primary implantation. The mean tumor volume was 1.53 ± 0.93 (SD) cm^3. Each tumor was excised completely and the wound was closed with surgical staples. The procedure was the same for each group except for the excisional modality used. In Group S, the tumor was excised with a scalpel. Hemostasis was achieved with electrocautery. The use of cautery was kept to a minimum. In Group E, excision was performed with a Surgistat electrocautery device (Valley Labs, Boulder, CO) with Coag set at 8 and Cut at 6.

In Group CS, tumors were excised with a Sharplan 1100 CO_2 laser (Sharplan, Allendale, NJ) at 25 W in continuous wave mode using a 125 mm handpiece in focus (0.2 mm spot size). Following excision, the entire wound surface was sterilized by defocusing the laser to a spot size of 5 mm and gently heating the tissue in a horizontal and then vertical pattern without blanching or charring. Tumors in group K were excised with a Laserscope KTP/532 laser (Laserscope, San Jose, CA) set at 17 W in continuous wave made. A 400 μm fiber was used. In Group Y, tumors were excised with a 0.2 mm clear laser scalpel tip and a Sharplan 2100 Nd:YAG laser set at 15 watts in continuous wave mode. A 600 μm coaxial gas-cooled fiber was used.

Surviving rats were examined for local primary tumor recurrence for 42 days following excision. All rats were implanted with a second tumor on day 14 following excision. Tumor pieces measuring 0.5 × 0.5 × 1.0 mm were implanted by trochar injection in the left flank. A control group of 13 naive unoperated female rats was also implanted with tumor in the mammary ridge. Growth of the secondary tumor was monitored via volume measurements for 36 days following implantation. Student's t-tests were done comparing the mean tumor volumes of the various groups.

TABLE 1. Local Recurrence f Primary Tumor

Group	N	No. of recurrences	% of recurrence
SCAL	13	3	23
ELEC	18	7	39
CO_2/STER	21	12	57
KTP	18	10	56
YAG	16	11	69*

*$P<0.05$ compared to SCAL.

RESULTS

The overall surgical mortality was 21%. The majority of deaths occurred in the scalpel group and can be attributed to hemorrhage. Electrocautery was used at a minimum in the scalpel group rats so as not to introduce a cautery-induced heat effect. This resulted in a higher mortality than in the laser and cautery groups were good hemostasis was achieved by the primary modality alone.

The rats were examined periodically for 42 days post-excision. The incidence of local tumor recurrence is presented in Table 1. The percentage of recurrence in the scalpel group (Group S) was significantly less than in the YAG laser group (Group Y), ($P < 0.05$, Chi-Square test). There were no other statistically significant dif-

TABLE 2. Mean Tumor Volume of Secondary Implants (cm^3)

Group	Days post-implantation 14	17	21	24	28	31	36	N
CTRL	0.60 ± 0.23	1.48 ± 0.54	2.74 ± 1.16	4.02 ± 1.82	5.62 ± 2.90	8.90 ± 4.23	14.86 ± 8.89	13
SCAL	0.55 ± 0.25	1.16 ± 0.61	1.74 ± 0.72*	2.87 ± 2.24	3.73 ± 2.83	6.23 ± 4.92	11.96 ± 8.53	11
ELEC	0.45 ± 0.30	0.96 ± 0.62	1.72 ± 0.99*	2.47 ± 1.38*	2.62 ± 1.68**	3.96 ± 2.70**	8.37 ± 5.02	17
CO_2/STER	0.38 ± 0.23	0.93 ± 0.64	1.50 ± 0.85**	2.03 ± 1.33**	2.54 ± 1.73**	3.99 ± 2.66**	8.98 ± 6.33	20
KTP	0.47 ± 0.20	1.15 ± 0.56	1.55 ± 0.76**	2.10 ± 1.06**	2.42 ± 1.31**	3.75 ± 1.50**	9.68 ± 4.93	16
YAG	0.43 ± 0.29	0.98 ± 0.48	1.48 ± 0.84**	1.98 ± 1.11**	2.57 ± 1.68**	3.95 ± 2.86**	10.35 ± 8.18	15

*$P < 0.05$ vs. CTRL.
**$P < 0.01$ vs. CTRL.

ferences. The majority of tumor recurrences were detectable by day 14 following excision. There were no additional recurrences observed after day 31.

Secondary tumor growth (tumor rechallenge) is presented in Table 2 and Figure 1. The mean tumor volume and its standard deviation are shown for each group on each day of measurement. Accurate assessment of tumor size was not possible until the 14th day following implantation. Six of the secondary implants did not grow and these rats were excluded from analysis. They were distributed as follows: two each in groups K and S, and one each in groups Y and E.

On days 21 through 31, the mean tumor volumes of the three laser and electrocautery groups were significantly less than control by t-test ($P < 0.01$). Scalpel was only significantly different from control on day 21 ($P < 0.05$). On the other days, the mean volume of the scalpel group was still less than control but the difference was not statistically significant. The mean volumes of the four thermal groups were always less than the scalpel group but this was never statistically significant. By day 36, there was no longer any difference among the groups.

Statistical analysis was also performed to determine whether the presence of a primary tumor recurrence had an effect on secondary tumor growth. In each surgical group, the rats were divided into those with a primary recurrence and those without. A t-test was performed comparing the secondary tumor volume in each pair of subgroups for each measurement day. In almost all cases, there was no significant difference found. The exceptions were in the electrocautery group on days 14 and 17 where the rats with primary recurrence had a significantly smaller secondary tumor volume. The subgroups without recurrence in the YAG and scalpel groups were significantly less on day 36.

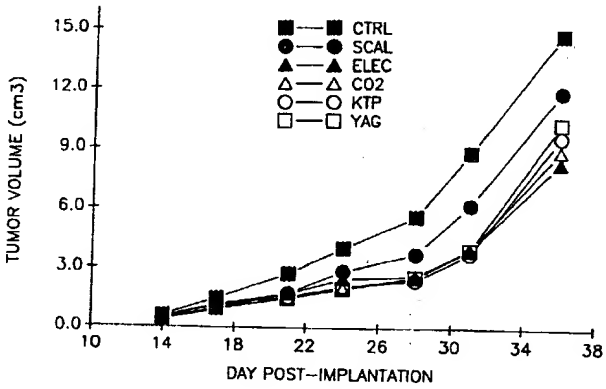

Fig. 1. The growth rate of secondary tumor implants is plotted as the mean tumor volume (cm^3) versus days post-implantation.

DISCUSSION

The results of this study conflict with those of previous work in our laboratory [1–5] from the perspective of primary tumor recurrence. In the previous studies, the scalpel group always recurred at a rate above 70%. Groups with laser excision followed by wound sterilization were always below a 38% recurrence rate. There are several possible explanations for this apparent discrepancy. The most important difference between this study and our previous work is that surgical excision was performed by a less-experienced surgeon (TPB). This stresses the importance of the learning curve with regard to laser technique and underscores the technique dependence of surgical outcome. The lower than expected recurrence in the scalpel group can be explained if a greater amount of normal tissue was excised surrounding the tumor than was accomplished with the laser. The higher than expected rate among the laser groups, particularly group CS, can be explained if small pieces of tumor were left behind due to less than optimal technique with laser excision and

sterilization. In these rats, recurrence would result from regrowth of these pieces rather than from undetected individual tumor cells (i.e., microscopic tumor rests). This is supported by the fact that the majority of recurrences were detected by day 14 following excision. This is the rate of growth at which trochar-injected pieces become clinically detectable. Individual cells would take longer to grow to a minimally detectable size.

This unexpected result as regards local recurrence does not alter the information from the secondary tumor challenge. Within each experimental group, the presence or absence of a primary recurrence had no effect on secondary tumor volume. This rules out any potential concomitant immunity. It appears that the local effects on tumor recurrence and the systemic host anti-tumor response are independent of one another. These two separate laser-induced effects may then operate through different mechanisms.

In terms of the secondary tumor growth, the experimental groups fall into three divisions. The unoperated control group clearly has the greatest tumor volume. The three laser and the electrocautery groups are of roughly equal tumor volume and make up the division with the smallest secondary tumor volume. The scalpel group is intermediate between the other two divisions with regard to secondary tumor volume.

Two conclusions can be drawn from this segregation of the experimental groups. First, surgery itself, regardless of modality, has some effect in augmenting the host anti-tumor response. This is demonstrated by the fact that secondary tumor growth is inhibited in all surgical groups, including the scalpel group, relative to the non-operated control group. The control group was implanted at a different site (mammary ridge) than the secondary tumor challenge in the excisional groups (left flank). But, this does not affect the results since there is no evidence that growth of the R3230AC tumor is implantation-site dependent. Secondly, excision with a laser or electrocautery serves to further increase the anti-tumor host response. The CO_2, YAG, and KTP lasers and cautery were more effective than the scalpel in reducing secondary tumor growth as measured by tumor volume. This corroborates the earlier studies with cryosurgery, tumor ligation, and electrocautery [6–10].

Six of the secondary implants did not grow and these rats were excluded from the analysis because it could not be determined why the implants failed to grow. It is probable that these implants were not placed properly or that these particular tumor pieces were not viable. A more intriguing and potentially more significant possibility is that the immune system completely rejected the secondary tumor in some of these rats as a result of laser-mediated immune augmentation. However, this explanation could not apply to all of the implants that did not grow as two of them were in the scalpel group and one in the electrocautery group.

There are several possible mechanisms by which laser excision could potentiate the host anti-tumor immune response. Tumor components released as a result of tumor cell bursting and vaporization could serve as antigenic stimuli for host immune cells. This would be a result of the thermal effects of the laser at the wound site. This thermal effect might also activate growth factors which could in turn activate immune cells. An increase in tumor antigenicity as a result of the altered wound inflammatory response following laser excision may be another possible mechanism. Lymphocytes activated by any of these mechanisms at the site of excision would then proliferate and circulate, thereby sensitizing the host to subsequent tumor challenge. Such a scenario may involve cytotoxic T cells, natural killer cells, and/or antibody produced by activated B cells.

Further studies are necessary to determine whether the host sensitization to subsequent tumor challenge is due to increased host anti-tumor immunity and to elucidate the mechanisms of this effect. Laser-induced immune modulation may have important clinical ramifications as regards both the control of tumor locally and the reduction of metastases.

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L30 ANSWER 28 OF 30 MEDLINE DUPLICATE 22
ACCESSION NUMBER: 95016440 MEDLINE
DOCUMENT NUMBER: 95016440 PubMed ID: 7931080
TITLE: Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin.
AUTHOR: Molloy A; Laochumroonvorapong P; Kaplan G
CORPORATE SOURCE: Department of Cellular Physiology and Immunology, Rockefeller University, New York 10021.
CONTRACT NUMBER: AI-07012 (NIAID)
AI-22616 (NIAID)
SOURCE: JOURNAL OF EXPERIMENTAL MEDICINE, **(1994 Oct 1)** 180 (4) 1499-509.
Journal code: 2985109R. ISSN: 0022-1007.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199411
ENTRY DATE: Entered STN: 19941222
Last Updated on STN: 19970203
Entered Medline: 19941102

AB We have examined the effect of killing of host monocytes infected with bacillus Calmette-Guerin (BCG) on the viability of the intracellular mycobacteria. Peripheral blood monocytes were infected in vitro with a single bacillus per cell and maintained in culture for 6-8 d to allow the bacilli to replicate. Replicating viable BCG were found singly in perinuclear vacuoles bounded by tightly apposed lipid bilayers. Monocytes were then exposed to toxic mediators that induced killing of cells as evaluated by 51Cr release into the culture medium. Both **hydrogen peroxide** (**H2O2**) (an inducer of cell **necrosis**) and adenosine triphosphate (ATP4-) (an inducer of cell apoptosis) treatment killed infected monocytes. H2O2-induced killing had no effect on BCG viability. ATP-induced cell death was accompanied by DNA fragmentation and nuclear condensation. Apoptosis was associated with a swelling of the phagocytic vacuoles which became multibacillary and with a reduction of BCG viability as enumerated by colony-forming units.

L48 ANSWER 5 OF 11 MEDLINE DUPLICATE 2

ACCESSION NUMBER: 87240214 MEDLINE
DOCUMENT NUMBER: 87240214 PubMed ID: 3036004
TITLE: Negative and positive assays of superoxide dismutase based on **hematoxylin** autoxidation.
AUTHOR: Martin J P Jr; Dailey M; Sugarman E
CONTRACT NUMBER: AI-19695 (NIAID)
SOURCE: ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, **(1987 Jun)** 255 (2) 329-36.
Journal code: 0372430. ISSN: 0003-9861.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198707
ENTRY DATE: Entered STN: 19900305
Last Updated on STN: 19970203
Entered Medline: 19870710

AB **Hematoxylin**, a natural dye commonly used as a histological stain, generates superoxide upon oxidation to its quinonoid product, hematein. The parameters affecting this reaction were assessed in developing a new and versatile assay for superoxide dismutase. The autoxidation of **hematoxylin** to hematein was accompanied by an increase in absorbance between 400 and 670 nm. The autoxidation rate was proportional to **hematoxylin** concentration and increased with pH above 6.55. Trace metals accelerated the autoxidation and this effect was eliminated by EDTA. Superoxide dismutase inhibited the autoxidation 90-95% below pH 7.8, but above pH 8.1 the rate was augmented by superoxide dismutase. The rate inhibition at low pH was proportional to the superoxide dismutase concentration up to 70% inhibition. The rate acceleration at high pH was proportional to superoxide dismutase concentration up to approximately 200% acceleration. The autoxidation rate was not significantly affected by ethanol, cyanide, azide, hydrogen peroxide, or catalase. However, the reaction was inhibited by the **reducing agents** NADH, reduced glutathione, ascorbate, and dithiothreitol, and by undialyzed extracts of Escherichia coli B. When cell extracts were dialyzed prior to assay, the degree of inhibition observed was proportional to the concentration of superoxide dismutase in the extract. These observations form the basis for negative and positive assays of superoxide dismutase which are inexpensive and simple to perform. The negative assay has the added advantage of being applicable at physiological pH.

L5 ANSWER 2 OF 3 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 94130271 MEDLINE
DOCUMENT NUMBER: 94130271 PubMed ID: 8299115
TITLE: Modification of monoclonal antibody carbohydrates by oxidation, conjugation, or deoxymannojirimycin does not interfere with antibody effector functions.
AUTHOR: Awwad M; Strome P G; Gilman S C; Axelrod H R
CORPORATE SOURCE: Department of Biological Research, CYTOGEN Corporation, Princeton, NJ 08540.
SOURCE: CANCER IMMUNOLOGY, IMMUNOTHERAPY, (1994 Jan) 38 (1) 23-30. Journal code: 8605732. ISSN: 0340-7004.
PUB. COUNTRY: GERMANY: Germany, Federal Republic of
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199403
ENTRY DATE: Entered STN: 19940318
Last Updated on STN: 19990129
Entered Medline: 19940308

AB Site-specific attachment of metal chelators or cytotoxic agents to the carbohydrate region of monoclonal antibodies results in clinically useful immunoconjugates [Doerr et al. (1991) Ann Surg 214: 118, Wynant et al. (1991) Prostate 18: 229]. Since the capacity of monoclonal antibodies (mAb) to mediate tumor cell lysis via antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) may accentuate the therapeutic effectiveness of immunoconjugates, we determined whether site-specific modification of mAb carbohydrates interfered with these functions. The chemical modifications examined consisted of periodate oxidation and subsequent conjugation to either a peptide linker/chelator (GYK-DTPA) or a cytotoxic drug (**doxorubicin adipic dihydrazide**).
mAb-associated carbohydrates were also modified metabolically by incubating hybridoma cells in the presence of a glucosidase inhibitor deoxymannojirimycin to produce high-mannose antibody. All four forms (unaltered, oxidized, conjugated and high-mannose) of murine mAb OVB-3 mediated tumor cell lysis via CDC. Similarly, equivalent ADCC was observed with native and conjugated forms of mAb OVB-3 and EGFR.1. ADCC was achieved with different murine effector cells such as naive (NS), poly (I*C)- and lipopolysaccharide-stimulated (SS) spleen cells, or Corynebacterium-parvum-elicited peritoneal cells (PEC). All murine effector cell types mediated tumor cell lysis but differed in potency such that PEC > SS > NS. Excellent ADCC activity was also demonstrable by human peripheral blood mononuclear cells with OVB-3-GYK-DTPA and high-mannose OVB-3 mAb. ADCC activity was detectable in vivo: both native and conjugated OVB-3 inhibited growth of OVCAR-3 xenografts in nude mice primed with C. parvum. In conclusion, modification of mAb carbohydrates did not compromise their in vivo or in vitro biological functions. Therefore, combination therapy using immunomodulators to enhance the effector functions of site-specific immunoconjugates could be seriously contemplated.

L8 ANSWER 6 OF 9 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1973:27700 CAPLUS
DOCUMENT NUMBER: 78:27700
TITLE: New method of preparation of specifically purified anti-**TNP** antibodies
AUTHOR(S): Lisowski, Jozef; Staroscik, Krystyna
CORPORATE SOURCE: Inst. Immunol. Exp. Ther., Pol. Acad. Sci., Wroclaw, Pol.
SOURCE: Journal of Immunological Methods (1972), 1(4), 341-52
CODEN: JIMMBG; ISSN: 0022-1759
DOCUMENT TYPE: Journal
LANGUAGE: English

AB A new method of prepn. of specifically purified rabbit anti-**TNP** antibodies is described, in which immunoadsorbents prepd. from **TNP** -bovine serum albumin (BSA), p-aminobenzyl cellulose (PAB-cellulose), and aminoethyl cellulose (AE-cellulose) are used. To increase efficiency, **TNP**-BSA was reduced and alkylated in 6M guanidine-HCl soln. before coupling with diazotized PAB-cellulose. For coupling with AE-cellulose in the presence of cyclohexyl(morpholinyl)**carbodiimide** no pretreatment of **TNP**-BSA is necessary. In the case of PAB-cellulose, 92 mg **TNP**-BSA was bound to 1 g of the cellulose carrier and, in the case of AE-cellulose, 100 mg of **TNP**-BSA was linked to 1 g of the cellulose. The best results were obtained with PAB-cellulose-**TNP**-BSA immunoadsorbent and 0.01M picric acid as eluting agent. With low affinity rabbit anti-**TNP** antibodies, 92% yield of 85-100% precipitable antibody was achieved. With other methods, only 12-48% yield of 50-90% precipitable antibodies was obtained.

L5 ANSWER 13 OF 51 MEDLINE DUPLICATE 8

ACCESSION NUMBER: 1999101035 MEDLINE
DOCUMENT NUMBER: 99101035 PubMed ID: 9885944
TITLE: Outcome after radiotherapy of primary spinal cord glial tumors.
AUTHOR: McLaughlin M P; Buatti J M; Marcus R B Jr; Maria B L; Mickle P J; Kedar A
CORPORATE SOURCE: Department of Radiation Oncology, University of Florida College of Medicine, Gainesville, USA.
SOURCE: RADIATION ONCOLOGY INVESTIGATIONS, **(1998)** 6 (6) 276-80.
Journal code: 9437448. ISSN: 1065-7541.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199903
ENTRY DATE: Entered STN: 19990324
Last Updated on STN: 19990324
Entered Medline: 19990311

AB Primary spinal cord tumors are rare, and **treatment** recommendations are therefore difficult. We reviewed a 22-year experience of postoperative radiotherapy for spinal cord tumors to elucidate prognostic factors and recommendations. Twenty-two patients with spinal cord tumors were treated from 1969-1991. Ten patients had ependymomas, of which two were high grade. Twelve had astrocytomas, of which 4 were high grade. **Karnofsky status**, age, extent of resection, tumor histology, grade, and radiation dose were evaluated, as well as degree of clinical improvement after **treatment** based on change in **Karnofsky status**. Ependymomas achieved 100% local control with postoperative radiotherapy. Grade and dose were of indeterminate significance because of these excellent results. High-grade astrocytomas all recurred and caused death. Disease recurred in 1 of 8 patients with low-grade astrocytic or mixed astrocytic tumors. The only prognostic variables of significance were histology, grade, and change in **Karnofsky status** after **treatment**. Radiation of primary spinal cord tumors is rare. In nearly all cases, local fields may be used. Improvement in **Karnofsky status** after radiotherapy may predict better survival. **Treatment** recommendations for these rare tumors are discussed.

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LONG-TERM TUMOR RESISTANCE INDUCED BY LASER PHOTO-IMMUNOTHERAPY

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An ideal treatment modality for metastasizing tumors should eradicate the primary tumor and elicit a systemic, tumor-selective response leading to elimination of metastases and long-term tumor resistance. Also, it should be induced by local treatment at the primary site, to limit adverse systemic effects. A new method for treating metastatic tumors which utilizes a combination of a near-infrared laser, a photosensitizer and an immunoadjuvant has been developed. It involves intra-tumor injection of the sensitizer/adjuvant solution, followed by local non-invasive laser irradiation. It has produced regression and total eradication of treated primary tumors and untreated metastases at remote sites against mammary tumors in rats. Successfully treated tumor-bearing rats showed total tumor resistance to subsequent tumor rechallenge. Our histochemical results showed that sera from cured tumor-bearing rats contained antibodies that bound strongly to the plasma membrane of both living and preserved tumor cells. Western blot analysis of tumor cell proteins using sera from successfully treated rats as the source of primary antibodies also showed distinct bands, indicating induction of tumor-selective antibodies. Our findings indicate that a systemic, long-term effect on metastatic tumors can be induced by local application of laser photo-immunotherapy. *Int. J. Cancer* 81:808–812, 1999.
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Systemic cure and long-term resistance to recurrence of malignant tumors of the same type have been elusive goals in the treatment of human cancer. Current systemic treatments, such as chemotherapy, have had limited success due to severe toxic side effects and a high rate of tumor recurrence. Even though single-modality treatments such as radiation and surgery can be curative locally, they lack systemic effects and fail to elicit long-term anti-tumor responses. Conventional immunotherapy depends on either cross reactive tumor-specific antigens or the use of non-specific immunological stimulation. The former has been unsuccessful because the ubiquitous tumor-specific antigens that can be isolated in all human tumors of the same type are not available; the latter, using immunotherapy with adjuvants such as bacille Calmette-Guérin (BCG), *C. parvum* and Freund's adjuvant, has also failed due to lack of specificity.

The use of lasers is gaining widespread acceptance as an effective local treatment due to the precision of energy delivery achieved with the assistance of photosensitizers, as in the case of photodynamic therapy (PDT) (Dougherty *et al.*, 1978, 1989; Fisher *et al.*, 1995). However, the immunological effects of PDT on cancer treatment are unclear. Some groups have reported increased activities of T cells and local macrophages following PDT (Canti *et al.*, 1994; Korbelik and Krosl, 1994; Krosl *et al.*, 1995, 1996), while others have reported immune suppression by PDT (Elmets and Bowen, 1986; Gomer *et al.*, 1988; Lynch *et al.*, 1989; Obochi *et al.*, 1995).

Laser immunotherapy (Chen *et al.*, 1997) was developed to achieve a systemic anti-tumor response in the treatment of cancers. It uses laser-dye photothermal tissue destruction and a co-administered immunoadjuvant for immune stimulation. Initial results indicate success in the treatment of metastatic mammary tumors in rats, including total eradication of primary tumors and untreated metastases. The mechanism of laser immunotherapy has been suggested to involve an induced tumor-selective immune response. The objectives of the current research are to further investigate the systemic effects of this treatment modality on metastatic tumors, to analyze the long-term tumor immunity induced by such treatment and to examine the possible immunological mechanisms using histochemical assays and Western blot analysis.

MATERIAL AND METHODS

Metastatic tumor model

DMBA-4 transplantable, metastatic mammary tumor cells (Kim, 1977) were implanted in young Wistar Furth female rats (Harlan Sprague-Dawley, Indianapolis, IN), ranging in age from 6 to 8 weeks and weighing 150 to 200 g. The tumor line was passed serially through living hosts in our laboratory. Rats were inoculated with 10^5 viable tumor cells s.c. in one of the inguinal fat pads 7 to 10 days before treatment. The primary tumor usually became palpable in 5 to 7 days, and the metastases in the remote inguinal and axillary areas appeared 15 to 20 days after inoculation. Without treatment, tumor-bearing animals had an average survival time of approximately 33 days.

Photosensitizer and immunoadjuvant

The photosensitizer used in our experiments was indocyanine green (ICG) (Becton-Dickinson, Cockeysville, MD). The immunoadjuvant used was glycated chitosan (GC) prepared in our laboratory by incubating an aqueous suspension of chitosan with a 3-fold excess of galactose and subsequent stabilization by borohydride reduction of the Schiff bases. Aqueous ICG solution has an absorption peak near 800 nm. The final aqueous solution contained 0.25% ICG and 1% GC. A volume of 200 µl of the solution was injected directly into the center of each primary tumor before laser treatment, resulting in an ICG dose of 2.5 mg/kg and a GC dose of 10 mg/kg.

Laser photo-immunotherapy in the treatment of metastatic rat mammary tumors

Laser treatment was performed when the primary tumors reached a size of 0.2 to 0.5 cm^3. Two hours after the ICG/GC intra-tumor injection, the tumor was irradiated using an 805-nm diode laser (Diomedics, Woodlands, TX) in a non-invasive mode. The tip of the optical fiber was maintained at a 4-mm distance from the skin overlying the tumor, and the fiber tip was moved smoothly over the entire tumor. A laser spot 3 mm in diameter was produced on the treatment surface. The laser was operated at 2 watts for 10 min, delivering a total energy of 1,200 J to the tumor. The total

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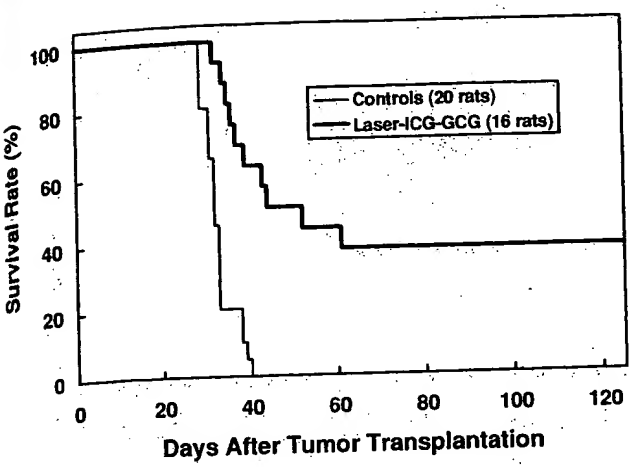

FIGURE 1 – Kaplan-Meier plot of rat survival rate after tumor inoculation. Thick curve represents 16 rats treated with an intra-tumor injection of 200 µl aqueous solution (0.25% ICG and 1% GC), followed by laser irradiation at 2 watts for 10 min. Thin curve represents 20 untreated control tumor-bearing rats, all of which died within 40 days of tumor inoculation, with an average survival time of 32.7 days (±3.5 days SD).

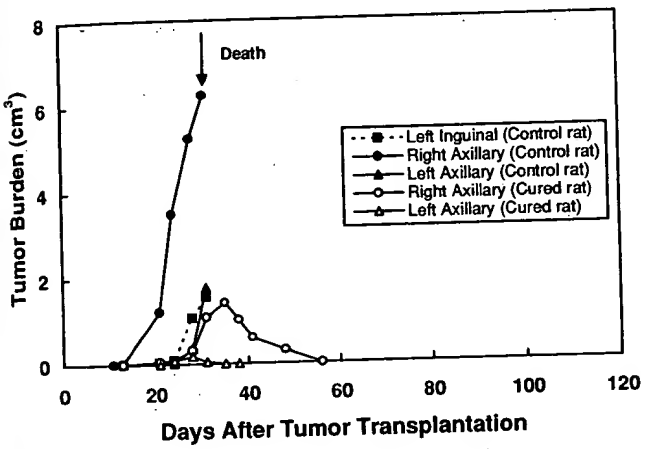

FIGURE 2 – Size of metastatic tumors in unimplanted axillary areas for a successfully treated tumor-bearing rat (open circles and triangles) and in the axillary and inguinal areas of an untreated control tumor-bearing rat (solid squares, circles and triangles). The primary tumor site in both cases was the right inguinal fat pat inoculated with 10^5 viable tumor cells. Laser–ICG–GC treatment took place on day 9. For the treated rat, the metastatic tumor burden continued to increase immediately following treatment, then began to decline between 30 and 35 days; the last metastatic tumor disappeared around day 55, and no recurrence was found throughout 240 days of observation. For the untreated rat, metastases continued to grow until the time of death on day 31, as noted by the arrow.

fluence delivered to each tumor was 68,000 J/cm² over the entire surface area.

Fluorescent labeling of living tumor cells

A tumor-bearing rat successfully treated by laser–ICG–GC was rechallenged with 10^6 viable tumor cells 120 days after the initial inoculation. Sera from the rechallenged rat (32 days after rechallenge) and from a control tumor-bearing rat were collected and diluted 1:1,000 in PBS. Freshly collected tumor tissue was dispersed to a single-cell suspension by grinding in a loose-fitting ground glass homogenizer. Approximately 10^6 tumor cells were incubated with 1 ml diluted serum for 1 hr at room temperature and

TABLE I – RECHALLENGE WITH TUMOR CELLS OF RATS PREVIOUSLY CURED BY LASER–ICG–GC TREATMENT

Group	Number of rats	Number of tumor cells	Tumor occurrence	Death rate (in 30 days)	Death rate (in 40 days)	Survival (days)
Cured rats[1]	15	10^6	0%	0%	0%	>120
Age-matched tumor control rats[2]	18	10^6	100%	83%	100%	28.2 ± 2.8
Young tumor control rats[3]	20	10^5	100%	20%	100%	32.7 ± 3.5

[1]Tumor-bearing rats cured by laser–ICG–GC treatment. These tumor-free rats were rechallenged with 10^6 viable tumor cells 120 days after the initial inoculation.–[2]Untreated rats of the same age as the cured rats at the time of inoculation, without previous exposure to tumor.–[3]Same tumor-bearing control rats shown in Figure 1, with tumor inoculation at the age of 8 weeks.

washed 3 times in PBS, which was followed each time by low-speed centrifugation, to remove unbound antibody. Cells were then incubated with secondary fluorescein-labeled goat anti-rat anti-serum (Sigma, St. Louis, MO) for 1 hr at room temperature and rinsed in PBS 3 times. Finally, cells were mounted in an aqueous mounting medium and viewed immediately with a fluorescence microscope.

Tumor tissue immunoperoxidase

Tumor tissue was fixed in 2% paraformaldehyde, then dehydrated and embedded in paraffin. Sections were cut, mounted on glass slides and then rehydrated. Sections were incubated for 1 hr with the diluted serum (1:1,000) from a control tumor-bearing rat and from a successfully treated tumor-bearing rat (32 days after tumor rechallenge), respectively. Sections were then rinsed 3 times in PBS. After the final wash, sections were labeled with peroxidase using an ABC kit (Vector, Burlingame, CA) and viewed by optical microscopy.

Western blot analysis

As the sources of primary antibodies, sera were collected from naive and untreated tumor-bearing rats, as well as from successfully treated tumor-bearing rats at different times after tumor rechallenge. Tumor tissue collected from a control tumor-bearing rat was homogenized to a single-cell suspension. Cells were washed twice in PBS at 4°C and then lysed in Laemmli's sample buffer (Bio-Rad, Hercules, CA) containing 5% β-mercaptoethanol at a final concentration of 8×10^6 cells/ml. Protein extract from 1.6×10^5 tumor cells was loaded into each well of a 10% SDS-polyacrylamide gel and electrophoresed at 100 V for 15 min and then at 200 V for 50 min. Proteins on the gels were transferred to a Hybond-ECL nitrocellulose membrane (Amersham, Arlington Heights, IL) with a 250-mA current at 4°C for 2.5 hr. The membrane was soaked in a blocking solution [50 mM Tris (pH 7.5), 0.9% NaCl, 0.05% Tween-20, 3% non-fat dry milk] for 2 hr at room temperature and incubated in a blocking solution containing rat serum (1:100) overnight at 4°C. After incubation, the membrane was washed 3 times and then incubated with the secondary antibody (anti-rat Ig, horseradish peroxidase–linked whole antibody; Amersham) in a 1:5,000 solution for 1 hr at 4°C. The membrane was then washed 3 times with blocking buffer and rinsed twice with cold PBS. Bands were visualized on X-ray film using a chemiluminescent detection system (Amersham) according to the manufacturer's specifications.

RESULTS

Long-term survival of tumor-bearing rats and regression of untreated metastases after laser immunotherapy

After treatment of primary tumors by laser–ICG–GC, increased survival and total tumor eradication were achieved. A recent experiment yielded 38% long-term survival 120 days after tumor

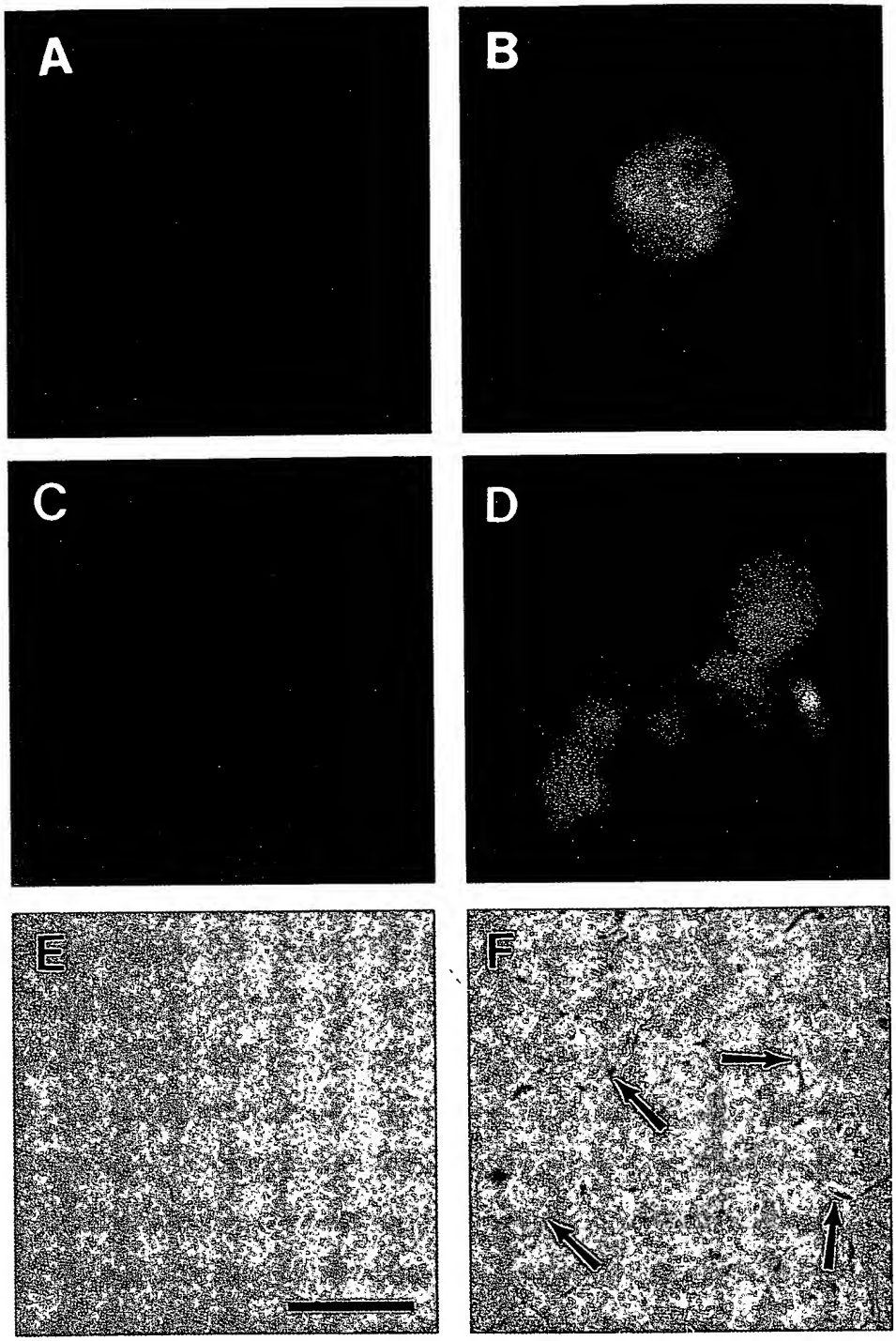

FIGURE 3 – Detection of tumor-selective antibodies in the sera of successfully treated tumor-bearing rats. *(a–d)* Representative photomicrographs of a single living tumor cell and a cluster of living cells incubated with sera from an untreated tumor-bearing rat 32 days after tumor inoculation *(a, c)* and from a successfully treated tumor-bearing rat 32 days after tumor rechallenge *(b, d)*. There is minimal fluorescence in the cell stained by serum from untreated tumor-bearing rats. In contrast, the tumor cell stained with sera from cured tumor-bearing rat shows greater fluorescence intensity and a uniform staining pattern over the plasma membrane. *(e, f)* Photomicrographs of tumor sections incubated with sera from an untreated tumor-bearing rat 32 days after tumor inoculation *(e)* and from a successfully treated tumor-bearing rat 32 days after tumor rechallenge *(f)*. Note in *(e)* the lack of brown reaction product that indicates peroxidase activity. In contrast, intense staining is seen in *(f)*. Note the intense staining at the plasma membrane (arrows) and the lack of staining within the cells. Scale bar: 20 μM.

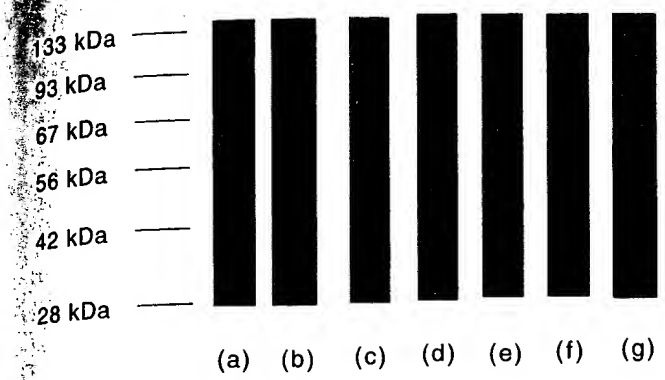

FIGURE 4 – Western blot analysis of tumor cell proteins using sera from different rats as the source of primary antibodies. *(a)* Tumor cell proteins probed using serum of a naive rat. *(b)* Tumor cell proteins probed using serum from an untreated control tumor-bearing rat. *(c–g)* Tumor cell proteins recognized using serum of a tumor-bearing rat cured by laser immunotherapy and 0 hr *(c)*, 1 week *(d)*, 2 weeks *(e)*, 1 month *(f)* and 2 months *(g)* after tumor rechallenge. There was very light antibody binding when the serum from the naive rat was used *(a)*. Note the differences in the 2 bands at approximately 45 and 35 kDa between the tumor control rat *(b)* and the cured rat *(c–g)*. There are also heavy bands at the bottom of the gel below 28 kDa (not shown) recognized by the serum from the cured rat.

inoculation, a 300% increase in the length of survival compared with untreated control tumor-bearing rats (Fig. 1). Although the primary tumors of long-term survival rats usually continued to grow immediately following treatment, after 4 to 6 weeks the tumor burden began to decrease and the tumor disappeared in 9 to 12 weeks. All tumor-bearing rats developed metastases in the remote inguinal and axillary areas 2 to 3 weeks after inoculation of the primary tumor. Untreated metastases of cured rats went through a regression pattern similar to that of the successfully treated primary tumors but with a much smaller burden and much earlier disappearance (Fig. 2). Using laser alone and the laser–ICG combination with the same parameters did not result in tumor eradication (data not shown).

Resistance to tumor rechallenge

To determine the long-term effect of laser immunotherapy, successfully treated rats were rechallenged with 10^6 viable cells, 10 times the number of tumor cells that created the original tumor. None of the successfully treated rats developed tumors. In contrast, all age-matched control tumor-bearing rats died within 35 days (Table I), with multiple tumors in the remote inguinal and axillary areas. Also shown in Table I are the data for 20 young control rats, inoculated with only 10^5 viable tumor cells; all of them developed multiple metastases but had a slightly increased survival time compared with the control rats inoculated with a higher tumor dose.

Antibody binding to tumor cells

After tumor rechallenge with an increased tumor dose (10^6 viable cells), sera obtained from successfully treated tumor-bearing rats were analyzed by 2 histochemical assays for tumor-selective antibodies. The first was an immunofluorescence assay which allows the detection of antibodies that bind to the plasma membrane of isolated live tumor cells. The second assay used preserved tumor tissue and the peroxidase reaction product to determine antibody binding to the plasma membrane and other cellular antigens. Both assays showed strong antibody binding using sera from successfully treated rats when compared with sera from untreated control tumor-bearing rats (Fig. 3).

Antibody binding to tumor cell proteins

Figure 4 shows Western blots of tumor cell proteins probed with sera from different animals: a naive rat, a control tumor-bearing rat and a successfully treated tumor-bearing rat. Apparently, the naive rat does not contain any tumor-selective antibody, as evidenced by the lack of staining in the Western blot (Fig. 4*a*). Sera from rats successfully treated by laser immunotherapy show 2 distinct bands (Fig. 4*c–g*) at approximately 45 and 35 kDa. The 45-kDa band was also observed after probing the blot containing the tumor proteins with serum from the control tumor-bearing rat but with much weaker intensity (Fig. 4*b*). The second band at 35 kDa was absent when the serum from the control tumor-bearing rat was used for probing.

DISCUSSION

Stimulation of a systemic and long-term anti-tumor response following localized tumor treatment was achieved using laser photo-immunotherapy. In this treatment modality, the laser and ICG provide a selective local photothermal reaction (Chen *et al.*, 1995*a,b,* 1996). Although ICG and the laser are known to yield a cytotoxic photochemical product, such as singlet oxygen (Fickweiler *et al.*, 1997; Reindl *et al.*, 1997), our results indicate that photothermal destruction of tumor tissue was the dominant reaction (Chen *et al.*, 1995*a,b,* 1996). Introducing the immunoadjuvant GC adds an immunological component to the treatment since chitosan stimulates an immune response in animals (Maeda *et al.*, 1992; Suziki *et al.*, 1986).

The tumor strain used in these experiments is highly aggressive; 99% of untreated tumor-bearing rats died with multiple metastases approximately 33 days after tumor cells were implanted (Table I). In rats successfully treated using laser–ICG–GC, total eradication of both primary and metastatic tumors and long-term resistance to tumor rechallenge were observed. We attribute these results to an induced immunological reaction. The effectiveness of this treatment is due to the immunoadjuvant GC since the photothermal effect of laser alone and of ICG alone does not result in long-term survival (Chen *et al.*, 1996). The tumor profile of the delayed tumor regression in successfully treated rats indicates an immune response intensified with time (Fig. 2). Furthermore, the regression of untreated metastases at remote sites indicates a systemic reaction. The resistance to tumor rechallenge in cured rats by laser immunotherapy strongly suggests tumor-selective immunity (Table I). Our histochemical assays using both live and preserved tumor cells detected tumor-selective antibodies in the sera of successfully treated tumor-bearing rats, at least a subset of which strongly bound to the plasma membrane of tumor cells (Fig. 3).

The characteristics of antibody binding to the tumor cell proteins in Western blot analysis also suggest a mechanism for the observed anti-tumor immunity induced by laser immunotherapy. Tumor cell proteins probed using the sera from successfully treated rats revealed several distinct bands. Particularly interesting are 2 bands at approximately 45 and 35 kDa (Fig. 4). When sera from tumor control rats were used, the 45-kDa band was observed with less intensity, while the 35-kDa band was absent (Fig. 4*b*). The 45-kDa band was absent when blots were incubated with sera from naive rats; it appeared only when rats were exposed to tumor cells and was enhanced markedly by laser immunotherapy (Fig. 4*c–g*). This may represent a natural host immune response to tumors, but without the laser immunotherapy, it would not be strong enough to control tumor growth. The 35-kDa band, however, suggests that a new antibody was induced in treated rats since this band was absent in naive and tumor control rats (Fig. 4*a,b*). The 35- and 45-kDa bands may represent specific immunodominant antigens.

We hypothesize that this tumor-selective immunity is the result of combined photothermal and photo-immunological interactions. The photothermal reaction reduces the tumor burden and at the same time exposes tumor antigens. The immune system, enhanced by the immunoadjuvant GC, would then recognize the exposed antigens and mount a systemic attack on the remaining cells of the treated tumor and on the untreated metastases. Without laser–ICG photothermal destruction, the immune system may not be able to

recognize the well-masked specific antigens on the tumor cell surface; without the immunoadjuvant stimulation, the host immune system may not react fast enough or reach the required strength to control the remaining mass of the primary tumor and its metastases, especially against the aggressive tumor model used in our studies.

Moreover, this immunity is induced by laser–ICG–GC in individual hosts bearing tumors through local treatment. This treatment could, in effect, produce an *in situ* autovaccine without the cross-reactive tumor antigens required by traditional immunotherapy.

Our previous study showed that without GC laser–ICG photothermal interaction could not have a curative effect (Chen *et al.*, 1996). Using laser alone with the same parameters was also inadequate in treating the metastatic tumors. Although laser–ICG treatment of tumor may have some immunological effect, it did not induce a detectable anti-tumor reaction under the conditions of our studies reported here.

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SPECIAL LECTURE SESSION

Possible Application of the Laser in Immunobiology

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Abstract. The human immune system acts a defence mechanism against exogenous or indigenous potentially harmful bodies, such as bacteria and viruses. The major histocompatibility complex (MHC class I and class II antigens) form key elements of legitimate body components, and the organization of MHC molecules allows T-lymphocytes to distinguish between legitimate and foreign bodies. On detection of a foreign component, T-cells activate the necessary pathways for destruction of the foreign body. Occasionally however the system breaks down and the result is a disease of an autoimmune nature. Both visible light and infrared low reactive-level laser therapy (LLLT) has been shown to act on immune system cells in a number of ways, activating the irradiated cells to a higher level of activity. Infrared LLLT has been shown to increase both the phagocytic and chemotactic activity of human leukocytes *in vitro*, for example. This is an example of photobiological activation. Photobiological cell-specific destruction is also possible using doses of low incident laser energy on cells which have been photosensitized for the specific wavelength of the laser, such as in photodynamic therapy (PDT) for superficial cancers. LLLT has also been shown to act directly and selectively on the autoimmune system, restoring immunocompetency to immunoincompetent cells. Although much more research needs to be done, there are enough experimental and clinical data to show that the laser, and LLLT in particular, has a possibly exciting role both in immunobiological therapy for diseases of the immune system, and to activate and boost the normal reaction of the immune system components against harmful foreign bodies. (Keio J Med 42 (4): 180–182, December 1993)

Key words: lasers, immune system

Introduction

The immune system of an individual can be defined as the defence mechanism to maintain the integrity of the individual (self) by the recognition and subsequent exclusion of unfamiliar exogenous and indigenous foreign substances (nonself) which originate or appear outside or inside the individual's body. To discriminate between self and nonself components, major histocompatibility complex (MHC) products, (class I and class II antigens), are employed as the key elements of self components, and T-lymphocytes can recognize self or nonself by observing how the self MHC molecules are organized. The MHC is a group of at least four linked loci, (A, B, C and D) on the sixth human chromosome collectively termed the human lymphocyte antigen (HLA) complex in man, which codes cells to produce histocompatibility antigens on the surface of cells.

Foreign substances or antigens are captured by antigen-presenting cells, processed, and presented on the cell surface together with class II antigens.

The role of the helped T-cell

When a specific type of thymocyte-derived lymphocyte, the helper T cell, recognizes the antigen and clas II complex as nonself, the helper T cells are activated to produce a variety of cytokines, nonantibody proteins such as lymphokines, which act as intercellular mediators for example in the generation of immune response. In addition to their function as effector cells in the production of delayed hypersensitivity, helper T cells exert their helper effects by the following two pathways. First, helper T cells facilitate the differentiation of B cells, which are already activated as antibody-production cells by the recognition of antigens as intact forms. Secondly, helper T cells are involved in the generation of cellular immunity, and the maturation of killer T precursor cells into killer T cells. Which pathway is preferentially induced depends on the nature of the antigens, but the

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effector cells thus produced are responsible for the removal of undesirable foreign substances from the body.

It should be pointed out however that the ability to distinguish self from nonself is not absolute. Occasionally the system breaks down and turns against the body, causing diseases of an autoimmune nature.

Laser Thepapy and The Immune System

Laser treatment can participate in the process of these immunological phenomena in two ways: Photobiological activation, and photobiological destruction. Photobiological activation can be defined as the mechanism whereby cellular function is normalized by means of light energy of very low intensity, and can be used to activate immunological responses nonspecifically. In contrast, photobiological destruction can be used to destroy cells at the molecular level, including the application of photodynamic therapy (PDT) employing a photosensitizer. Therfore, we should consider carfully the wavelength of the laser and its absorption rate in the target cells: and trials should be focused on those cells particularly disadvantageous to the body. In this case, it is recommended to heighten the photoselectivity of the target cells by using a cell-specific phtosensitizer at the molecular level. These types of photobiological reaction are applicable in the immunological field in both *in vitro* or *in vivo* treatment, for *in vivo* application, however, an appropriate irradiation methodology must be devised due to the high rate of absorption by the superficial tissues in the body. *In vivo* modulation of immunological responses has been achieved so far by; (1) irradiation of the superficial tissue (skin, mucosa, etcetera); (2) extracorporeal irradiation od circulating blood cells; and (3) interstitial fibreoptic irradiation of deeply located tissue.

Clinical Data

Preliminary clinical data from Skobelkin based on external infrared LLLT irradiation of specific sites in cancer patients, such as tumour projections and lymph nodes show a strong immunological system response.[1] Skobelkin's group performed preoperative LLLT on selected cancer patients undergoing palliative surgery. The levels of T-lymphocytes, T-helpers and T-suppressors were assayed for the 7 days following the operation, as were immunoglobulin levels, specifically IgA, IgM and IgG. The levels of blood-borne leukocytes, lymphocytes and monocytes all rose after laser therapy. Significantly increased levels of activated T-lymphocytes and helper T-cells were seen, with a significantly lower number of T-suppressors especially by the fifth post-therapy day. Increased levels of IgA and IgG were seen by the second post-therapy day, with a sharp reduction to almost normal levels by the fifth day. IgM levels rose slowly over the first four days, then rose sharply on the fifth day and maintained a high level on the subsequent levels during the period of the study. Skobelkin proposed that these were all indications of a strong photoactivated immunological response, with boosting of the competency of the somewhat immunoincompetent systems of these long-term cancer patients. The high levels of IgG, especially cytotoxic for tumoural cells, has also been associated with a corresponding rise in killer T-cells.[1] The antigen which would normally trigger these reactions was shown to be absent in all patients, thus the reaction was entirely photoactivated. Skobelkin did not report any activation of tumoural remnants following LLLT, which has been of major concern to many researchers.

These clinical data have been confirmed in a number of *in vivo* and *in vivo* studies using animal models by Dima,[2] Karu (Laser Therapy, in press) and Abe (Laser Therapy, accepted for publication). The studies all involve implanted virile carcinomas in rat and mice models, and they all have the result in common that the life span of the LLLT-irradiated animals was increased from 50% to 95% compared with unirradiated control animals. Some animals in Abe's study actually survived. All studies report increased levels of activated T-lymphocytes, T-helper and T-killer cells, in addition to increased immunoglobulin concentrations. Karu further showed that irradiating immune system cells with a normal level of competency had no effect, whereas the levels of competency of immunoincompetent cells were restored at least partially and at best completely. There are thus two LLLT-mediated mechanisms involved, according to these data: Photoactivation of the immune system processes which increase the level of immune response, and a specific cell-selective response in immunoincompetent cells.

Data from Osanai *et al*[3] showed in an *in vitro* study that specific doses of infrared LLLT increased the phagocytic activity of pooled human neutrophils as assayed by the luminol-dependent chemiluminescence method. Chemotaxis, the chemically-motivated tendency of the neutrophil to move towards an attractant foreign body prior to phagocytosis, was also increased in the LLLT-irradiated cells compared with unirradiated controls.

Conclusions

Although much more detailed research is necessary, especially for *in vivo* studies, the early data on LLLT action on the immune system specifically in cancer-infected systems is very promising. It is clear from all the reports that *in vivo* LLLT dose not activate the tumour cells to reproduce faster, but in fact inhibits growth and increases cell-specific destruction. This is one case where *in vitro* studies might well be misleading, as *in vitro*

cancer cells are removed from their immune system mediated environment, and might well show signs of enhanced reproduction. Other immune system-related diseases, such as atopic dermatitis, some forms of eczema, asthma and asthma-related ulceration, have responded well to LLLT.[4] In the case of atopic dermatitis, irradiation of only one affected site effected a systemic cure, lending credence to the systemic effect of LLLT on the immune system components. The future of LLLT applications in many immune system diseases and conditions, given the necessary research, is certainly promising and exciting.

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ORIGINAL ARTICLE

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Intracranial malignant lymphomas: clinicopathological study of 26 autopsy cases

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Abstract We examined 26 autopsy-proven cases of intracranial malignant lymphoma (IML) in immunocompetent patients to determine the extent of neoplastic involvement of the central nervous system (CNS) and to evaluate the effects of radiation on the tumor and brain tissue. All tumors were identified as diffuse non-Hodgkin's lymphomas of B-cell origin. In six patients who had not received radiotherapy, the clinical course of the disease was short and extensive infiltration of the tumor was seen. The remaining 20 patients were treated with radiotherapy and had a longer survival time. Leptomeningeal involvement was common, but extensive subarachnoid proliferation of the tumor was seen in only two cases. The posterior, but not anterior, lobe of the pituitary was involved in 5 of 22 cases, and choroid plexus involvement was seen in 4 of 21. Direct invasion of the tumor into the spinal cord, which tended to occur in patients with posterior fossa masses, was observed in 5 of 21 cases. Following irradiation, coagulation necrosis was frequently found in the invading zone as well as in the tumor mass, and degeneration of the white matter was also seen. We suggest that IML can extensively infiltrate into the CNS, including the posterior lobe of the pituitary and spinal cord, and that radiation injury to the brain appears to occur relatively easily in this disease.

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Key words Lymphoma · Central nervous system · Autopsy · Irradiation

Introduction

An increase in the incidence of primary intracranial malignant lymphoma (IML) has recently been reported in immunocompetent individuals.[1,2] Current treatment regimens for this disease are, however, unsatisfactory.[3] The best results reported to date have been achieved by administration of systemic and intra-Ommaya methotrexate, followed by plus boost radiotherapy of the whole brain and high-dose cytarabine after irradiation, yielding a median survival time of 42.5 months.[4]

Previous neuropathological investigations have revealed that the majority of central nervous system (CNS) lymphomas are diffuse non-Hodgkin's neoplasms of B-cell origin,[3,5–10] which proliferate and spread in the brain.[5,9,11–16] However, few studies have shown neoplastic involvement of the entire CNS, including the pituitary gland and spinal cord, or given detailed clinical data. Moreover, there has been a paucity of comparative investigations of irradiated and non-irradiated IML-bearing brains based on relatively large series, which would be helpful for reconfirming the role of radiotherapy in the treatment of the disease.

We carried out a clinicopathological examination of brain tissue taken postmortem from 26 patients with IML in order to determine the topographic involvement of the CNS and evaluate the effects of irradiation on the tumor and brain tissue.

Materials and methods

Patients

Between 1964 and 1995, autopsies were performed on 26 patients who had originally presented with neurological symptoms (Table 1). Seven of these patients have been reported previously.[17] Ten patients (cases 1–5, 7–10, and 19)

Table 1. Clinical summary of patients with intracranial malignant lymphoma

Case	Age/sex[a]	Primary site[b]	Surgery[c]	Histology[d]	Radiation (Gy)[e]	Chemotherapy[f]	Survival[g]
1	52/M	Lt thalamus‡	CVD, VPS	DSM/B	–	–	2 (7)
2	63/M	Bitemporal‡	–	DLI/B	–	P	2 (3)
3	57/F	Lt CP angle‡	CVD, Bx, VPS	DSC/B	–	D, P	3 (6)
4	43/F	Rt basal ggl‡	D	DL/B	–	MMC	2 (4)
5	42/M	Rt thalamus‡	D	DSC/B	–	B	1 (2)
6	69/M	Lt temporoparietal	Bx	DL/B	–	MVP	5 (6)
7	39/M	Brainstem‡	–	DSM/B	42†	P	6 (9)
8	50/M	Rt temporal‡	D, Bx	DSC/B	50†	B	6 (6)
9	32/M	Rt frontal‡	D + GTR, *CVD, VPS, PR*	DSC/B	40†	C, *P*	7 (9)
10	11/F	Lt frontal‡	D + Bx, PR	DSC/B	60†	VEMP	6 (10)
11	55/M	Lt parietal	D + Bx	DL/B	49†, *15*†, *15*†, *16*†	VABP, *VABP*	12 (12)
12	55/F	Cbll	PR, *GTR*	DL/B	36* + 20† + 30§, *30*†	P, *P*	138 (139)
13	62/F	Rt CP angle	–	DL/B	36* + 18† + 50§	P	6 (12)
14	69/M	Bifrontal	–	DL/B	40* + 14†	P, *P*	10 (11)
15	59/F	Lt frontal	D + GTR	DL/B	44* + 10†, *20*†, *46*§	*P*	29 (30)
16	42/F	Rt thalamus	Bx	DL/B	36* + 18†, *6**	MVP, *CHOP + B*	28 (29)
17	55/M	Brainstem, cbll	Bx	DL/B	50* + 31§	MVP	4 (5)
18	45/F	Lt cbll	Bx	DSM/B	35* + 18†	MVP	17 (18)
19	58/M	Lt parietal‡	STR	DL/B	48†		8 (9)
20	71/M	Lt cbll	PR	DL/B	24*	P	2 (3)
21	24/F	Rt temporothalamic	D, *STR*	DL/B	39* – 10†, *30*†, *36*†, *20*†, *16**	D, VABP + T, *VABP + T*	63 (67)
22	48/M	Lt frontal, thalamus	D + Bx, *CVD*	DSC/B	41* + 13† + 40§, *8*†	VABP, *CHOP*	28 (29)
23	51/M	Bifrontal, basal ggl	*Bx*	DSC/B	35* + 16† + 30§, *20*†	P, *P, CEMe, MVEMe*	91 (92)
24	70/M	Rt parietal	Bx	DL/B	36*	VP	2 (3)
25	65/M	Callosal	Bx	DL/B	24* + 34†	MVP	3 (3)
26	52/F	Bifrontal	Bx	DL/B	46* + 11†	MVP	20 (21)

[a] Age at first admission

[b] ‡ Angiography or ventriculography suggested the sites in these cases

[c] CVD, Continuous ventricular drainage; VPS, ventriculoperitoneal shunt; Bx, biopsy; D, decompressive craniectomy; GTR, gross total removal; PR, partial removal; STR, subtotal removal
Italics indicate operations at recurrence

[d] DSM, Diffuse small mixed; DLI, diffuse large immunoblastic; DSC, diffuse small cleaved; DL, diffuse large; B, B-cell lymphoma

[e] † Local irradiation. * Whole-brain irradiation. § Spinal irradiation
Italics indicate irradiation at recurrence

[f] P, Prednisone; D, dexamethasone; MMC, mitomycin C; B, Bleomycin; MVP or VP, vincristine + prednisone with or without methotrexate; C, cyclophosphamide; VEMP, vincristine + endoxan + methotrexate + prednisone; VABP, vincristine + ACNU + bleomycin + prednisone; CHOP, cyclophosphamide + adriamycin + vincristine + prednisone; T, tegafur; CEMe, CDDP + etoposide + methylprednisone; MVEMe, methotrexate – vincristine + etoposide + methylprednisone
Italics indicate chemotherapy at recurrence

[g] Months from the first admission until death. Parentheses indicate months from onset of neurological symptoms until death

were hospitalized for treatment before the introduction of computed tomography. Patients 13 and 17 developed signs of spinal involvement during the course of cranial irradiation and then received radiotherapy directly to the cord. Patient 15 had recurrence with signs of spinal involvement and received spinal irradiation. In patient 12, histological examination led to a misdiagnosis of medulloblastoma, which resulted in whole-neuraxis irradiation. Patients 22 and 23 underwent prophylactic irradiation of the spinal cord because of positive cerebrospinal fluid (CSF) cytology. The clinical details of these patients are summarized in Table 1.

All 26 patients were subjected to neuropathological examination of the CNS post-mortem.[18] In 15 patients, the entire CNS and visceral organs were examined, whereas in the remaining 11 patients, autopsy was limited to the intracranial cavity.

Histological examination

Tissues were fixed in 4% paraformaldehyde and, after gross examination, the following blocks were embedded in paraffin for routine histopathological examination: bilateral frontal, temporal, parietal, and occipital lobes; bilateral thalami, caudate nuclei, internal capsules, lenticular nuclei, callosal bodies, fornices, choroid plexuses, pituitary glands, midbrain, pons, cerebellum, and medulla oblongata. In addition, the cervical, thoracic, and lumbar spinal cord, as well as visceral organs including the lymph nodes and bone marrow, were examined in the complete autopsies. Sections were cut and stained with hematoxylin and eosin (H&E) and by Klüver-Barrera (K-B) method. Selected sections were also stained with Bodian's, periodic acid-Schiff, and Mallory-azan stain. For histological classification, autopsy material and biopsy specimens, when available, were examined and classified according to the Working Formulation.[19]

Immunohistochemistry

Immunophenotypes were investigated using markers for B cells (L-26; Dakopatts, Glostrup, Denmark) diluted 1:100 and T cells (UCHL-1; Dakopatts) diluted 1:100. Immunostaining was performed using the avidin-biotin-peroxidase

complex (ABC) method with a Vectastain ABC kit (Vector, Burlingame, CA, USA).

Results

Tumor classification

Fifteen of the 26 tumors were diffuse large-cell type, 7 were diffuse small cleaved-cell type, 3 were diffuse mixed-cell type, and 1 was diffuse large-cell immunoblastic type (Table 1). All 26 tumors were L-26-positive and UCHL-1-negative B-cell neoplasms.

Distribution of lymphoma cells in the CNS

The distribution of the lymphoma cells in the CNS is summarized in Table 2. The 26 cases were divided into two groups on the basis of radiotherapy.

Group 1: not treated with radiotherapy (cases 1–6)

These patients were treated with palliative surgery and/or minimal chemotherapy (Table 1). Five did not survive more than 3 months after admission. IML was the cause of death in all patients.

Case 3 showed diffuse subarachnoid proliferation with minimal parenchymal invasion of the tumor around the brain and spinal cord, compatible with so-called primary leptomeningeal lymphoma (Fig. 1). In the other five cases, lymphoma cells were spread widely in the cerebrum, brainstem, and cerebellum, and localized leptomeningeal invasion was also observed. The posterior, but not the anterior, lobe of the pituitary was involved in two cases, and choroid plexus involvement (Fig. 2) was seen in one case. However, no spinal infiltration was observed. Systemic involvement was not detected in four complete autopsy cases (Table 1).

Group 2: treated with radiotherapy (cases 7–26)

Twelve cases (cases 7–18) were treated with radiotherapy but had recurrent or residual lymphoma cells in the CNS at autopsy. Death was due mainly to CNS lymphoma. As in group 1, the brainstem and cerebellum were frequently involved. In addition, the cerebrum was more intensely invaded than in group 1. Five cases showed spinal involvement of the tumor at autopsy. In case 13, lymphoma cells proliferated more intensely in the subarachnoid space than in the parenchyma in all levels of the spinal cord. Cases 7, 10, and 17 had intramedullary infiltration without meningeal involvement, and case 11 had an intramedullary tumor with very slight local meningeal invasion of the cervical and thoracic cord. In cases 7 and 11, the cervical cord was more intensely invaded than the thoracic cord (Figs. 3 and 4). Localized meningeal invasion was seen in all cases, only one of which showed diffuse subarachnoid growth of the tumor cells (case 13). Involvement of the choroid plexus and posterior lobe of the pituitary (Fig. 5) was also observed occasionally.

In two cases (cases 19 and 20), systemic involvement was detected at autopsy. Patient 19 developed skin tumors 5 months after initial treatment for the brain lymphoma. At autopsy, tumor involvement was found in the skin, both lungs, cervical lymph nodes, pericardium, stomach, cecum, mesentery, renal pelves, and right deferent duct. Tumor cells were scattered throughout the brain. In case 20, lymphoma cells had invaded the right lung – with the potential to cause fatal pneumonia – liver, left adrenal gland, and left paraaortic lymph nodes, as well as the leptomeninges and cerebellum.

Six patients given radiotherapy (cases 21–26) had no, or few, residual tumor cells at autopsy. In these cases, no tumor cells were identifiable microscopically except in case 21, where a few residual neoplastic cells were evident in the subarachnoid space (Table 2). The cause of death appeared to be radiation-induced brain injury in cases 21, 22, and 26, pneumonia in cases 24 and 25, and chemotherapeutic myelosuppression in case 23.

Necrotic parenchymal lesions in areas of nonirradiated brain

In cases 1, 4, 5, and 6 of group 1, foci of necrosis were observed in areas with tumor infiltration (Fig. 6). Histologically, these areas displayed well-demarcated lesions showing tissue rarefaction, astrogliosis, and loss of myelin and axons. These lesions appeared to be ischemic in nature.

Coagulation necrosis and white matter degeneration in the irradiated brain

In the irradiated brains, coagulation necrosis was almost always observed in the tumor mass lesions and infiltrating zones. These necrotic foci contained many foamy macrophages. Blood vessels showing hyaline thickening were observed in or around the necrotic lesions (Fig. 7). In addition, degeneration of the white matter, seen as areas with indefinite borders, with loss and destruction of myelin and axons, was a common finding (Fig. 8). These features were observed in group 2 but not in group 1.

CSF cytology

Positive cytology was recorded in cases 1, 5, 11, 12, 14, 17, and 21–23, whereas cases 3, 4, 6, 10, 13, 15, 16, 24, and 25 had negative cytology.

Discussion

The data from this study confirm that the majority of IML cases are diffuse non-Hodgkin's neoplasms of B-cell origin. The histological subtypes of these tumors have recently been classified according to the Working Formulation or Kiel classification.[20] In various studies, the relative inci-

Table 2. Distribution of lymphoma cells at autopsy

Case	Visceral organs	Spinal cord[a]			Medulla oblongata	Pons	Cbll	Midbrain	Choroid plexus[b]		Pituitary gland[c]	Leptomeninges[d]
		L	Th	C					4th	Lateral		
1	−	−	−	−	−	+	+	+	−	−	NE	+ (L)
2	−	NE	NE	NE	+	+	+	−	NE	NE		+ (L)
3	−	−	−	−			−	−	−	−	+ (P)	+ (D)
4	NE	NE	NE	−	−	+	−	+	−	−	NE	+ (L)
5	NE	NE	NE	NE	−	+	+	+	NE	−	NE	+ (L)
6	−	−	−	−	+	+	+	+	+	+	+ (P)	+ (L)
7	−	−	+	+	+	+	+	+	NE	NE	−	+ (L)
8	NE	NE	NE	NE	+	+	+	+	NE	−	−	+ (L)
9	NE	NE	NE	−	−	+	+	+	−	−	−	+ (L)
10	NE	NE	NE	+	+	+	+	+	+	+	− (P)	+ (L)
11	−	−	+	+	+	+	+	+	NE	NE		+ (L)
12	NE	NE	NE	NE	+	+	+	+	+	NE	−	+ (L)
13		−	−	+	+	+	+	+	+	+	−	+ (D)
14		−	−	−	+	+	+	+	−	NE	+ (P)	+ (L)
15	NE	NE	NE	−	+	+	−	+	NE	NE	−	+ (L)
16	NE	NE	NE	−	+	+	+	+	−	−	−	+ (L)
17	−	−	−	+	+	+	+	+	NE	−	+ (P)	+ (L)
18	NE	NE	NE	NE	+	+	+	+		−	−	+ (L)
19	+	−	−	−	+	−	−	+	NE	NE	NE	+ (L)
20	+	−	−	−	−	−	+	−	−	−	−	+ (L)
21	−	−	−	−	−	−	−	−	−	NE	−	+ (L)
22	NE	NE	−	−	−	−	−	−	−	−	−	
23	NE	NE	NE					−			−	−
24	−	−	−	−	−	−	−	−	NE	−	−	−
25	−	−	−	−	−	−	−	−	−	−	−	−
26	−	−	−	−	−	−	−	−	−	−	−	−

+, Tumor positive; −, tumor negative; NE, not examined
[a] L, Lumbar; Th, thoracic; C, cervical
[b] 4th, Fourth ventricle; Lateral, lateral ventricles
[c] (P) Posterior lobe of the pituitary
[d] (L) Local meningeal invasion; (D) diffuse subarachnoid proliferation

dences of diffuse large-cell, diffuse large-cell immunoblastic, diffuse small cleaved-cell, small non-cleaved-cell (non-Burkitt's), diffuse mixed-cell, and atypical or unclassified tumors have been reported to be 43.4%, 19.7%, 9.5%, 8.8%, 7.1%, and 7.1%, respectively.[2] In our study, the relative frequencies of diffuse large-cell, diffuse small cleaved-cell, diffuse mixed-cell, and diffuse large-cell immunoblastic tumors were 57.7%, 26.9%, 11.5%, and 3.8%, respectively. There have been several previous reports documenting a relatively high frequency of tumors of the diffuse small-cell subtype[6] and a low incidence of tumors of the diffuse large-cell immunoblastic subtype,[8,15] which corresponds to the distribution observed in the present study. The difference in incidence of the various tumor subtypes between studies underlines the difficulty in subtyping IML using current histological criteria.[2] A new classification system,[21] which adopts immunohistochemical and genetic criteria, has recently been proposed, and this may help to overcome this problem.

IML is described as showing multifocal and diffuse spread, a perivascular arrangement of the tumor cells, secondary features such as subpial and subependymal spread, and frequent invasion of the leptomeninges.[2,14,16] These features were also seen in our patients. Tumor infiltration was more prominent in the white than in the gray matter, and the extent of tumor infiltration in the CNS appeared to be related to survival time. Twelve patients in group 2 (cases 7–18), who underwent radiotherapy and lived longer than those in group 1, had more extensive infiltration of neoplastic cells at autopsy. It is possible that the lymphoma cells had nearly all been killed by the radiotherapy, but that the surviving cells then proliferated in situ and spread. In some cases in group 2, antineoplastic treatment was not indicated at recurrence because of the poor neurological condition of the patient. This might also have made it easier for the lymphoma to infiltrate.

To our knowledge, there have been few reports documenting the involvement of the pituitary gland in IML.[14,16] In our series, exclusive involvement of the posterior lobe of the pituitary appeared to be common. The presence of highly infiltrative lymphomas in brain regions, including the hypothalamus, suggests that the tumor spreads via the pituitary stalk. Although diabetes insipidus was not clinically evident in these patients, it is possible that this condition was present.

Spinal cord involvement is not a rare event in CNS lymphomas.[14,22,23] Our observations suggest that intraparenchymal spread from the medulla oblongata down to the cervical and, in a few cases, the thoracic cord is the principal route of tumor spread. Although spinal cord involvement was seen in two patients (cases 10 and 11) with supratentorial tumors at presentation, it was observed more

Thalami		Caudate nuclei		Internal capsule		Lenticular nuclei		Callosal body	Fornix	Frontal lobes		Parietal lobes		Temporal lobes		Occipital lobes	
Rt	Lt	Rt	Lt	Rt	Lt	Rt	Lt			Rt	Lt	Rt	Lt	Rt	Lt	Rt	Lt
+	+	+	+	+	+	+	+	−	−	+	−	−	−	+	−	−	−
NE	NE	−	N	+	NE	+	NE	NE	NE	NE	NE	NE	NE	+	+	NE	NE
−	−	−			−	−	−	−	−	−	−	−	−	−	−	−	−
+		+	+	+	+	+	−	+	−	+	+	+	−	+	+	+	+
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+	+	+	+	+	+	+	+	+	NE	+	+	+	+	+	+	+	+
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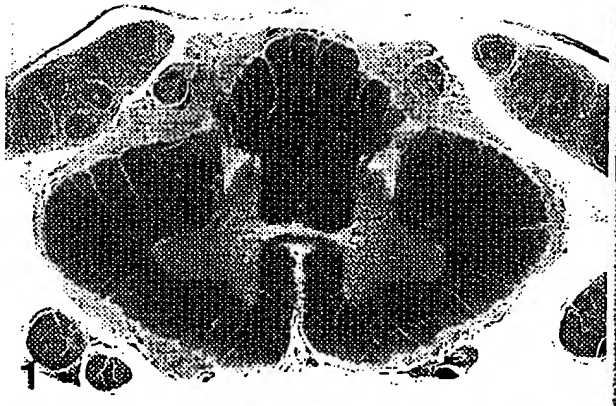
frequently in patients with primary tumors in the posterior fossa (cases 7, 13, and 17). Infratentorial lymphomas were found in seven of our patients (cases 3, 7, 12, 13, 17, 18 and 20). Patients 12 and 18 did not undergo cord examination, and patient 3 had a primary leptomeningeal lymphoma. Among the remaining four cases, three (75%) had spinal involvement. Thus, it is inferred that infratentorial malignant lymphomas have a high probability of spinal infiltration during the course of the disease. If radiation therapy is indicated for the treatment of brain lymphomas with posterior fossa masses, we recommend irradiation of the entire cranial contents and cervical cord.

This study supports the argument that invasion of the leptomeninges is frequent in IML and that extensive tumor spread throughout the subarachnoid space also occurs occasionally.[9,14] The leptomeningeal involvement is usually local and does not appear to result in CSF dissemination, since diffuse subarachnoid proliferation of the tumor occurred in only one (case 13) of the 20 (5%) cases with local meningeal invasion. The high frequency of positive CSF cytology,[24] together with our autopsy findings, suggests that the lymphomas commonly involve the leptomeninges and often shed tumor cells into the CSF. However, neoplastic cells are rarely found at distant sites in the subarachnoid space. In addition, occasional involvement of the choroid plexus might results from local invasion of the tela choroidea.

The necrotic lesions observed in areas with tumor infiltration were similar histologically to those seen in the spinal cord of patients with disseminated glioblastomas reported previously.[18] Perivascular proliferation of IML, which appears to affect regional circulation, could be observed at the periphery of the mass. Thus, the invading zone is potentially ischemic and tends to become necrotic as the infiltration progresses further. This circulatory condition may also be important in the development of radiation-induced brain necrosis in this disease. In our patients, when radiotherapy resulted in tumor necrosis, the neighboring brain tissue also became necrotic.[17] The necrotic areas contained thickened, hyalinous vessels similar to those seen in delayed radiation necrosis of the brain. On the other hand, destruction of myelin and axons in the white matter has also been noticed in irradiated brains.[25,29] This degenerative change was observed exclusively in irradiated brains, irrespective of tumor infiltration. These findings suggest that radiation injury to the brain may occur relatively easily in patients with CNS lymphomas. Alternatively, repeated radiotherapy could have contributed to the radiation injury in several of our cases. It is also known that administration of chemotherapy, especially methotrexate, after brain irradiation is associated with leukoencephalopathy.[30] Thus post-irradiation chemotherapy might have some influence on the pathological findings. In any event, a reduction of the radiation dose seems warranted for this disease.[31]

Acknowledgments The authors would like to thank the neurosurgeons and neuropathologists who contributed to this study, in particular Drs.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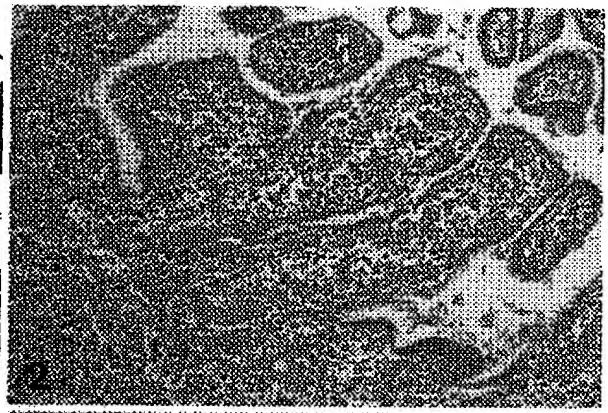
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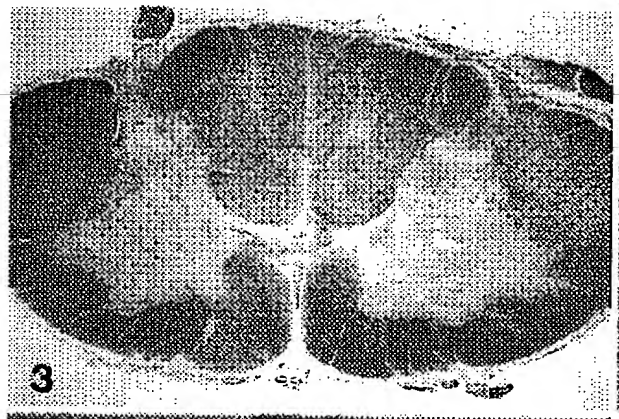
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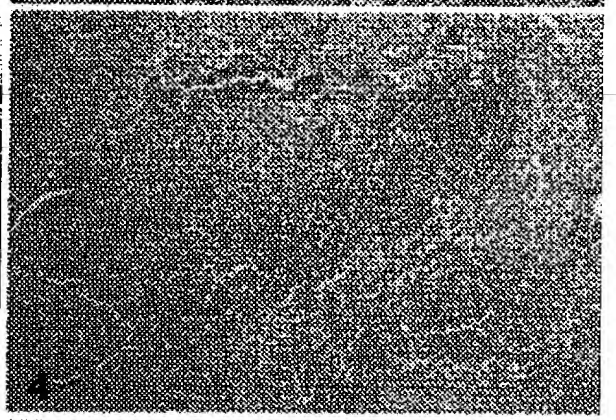
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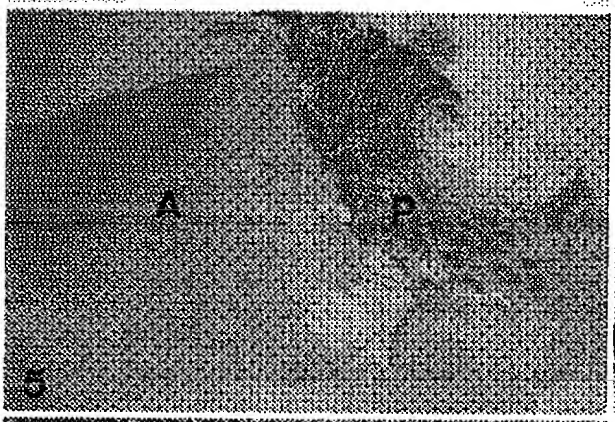
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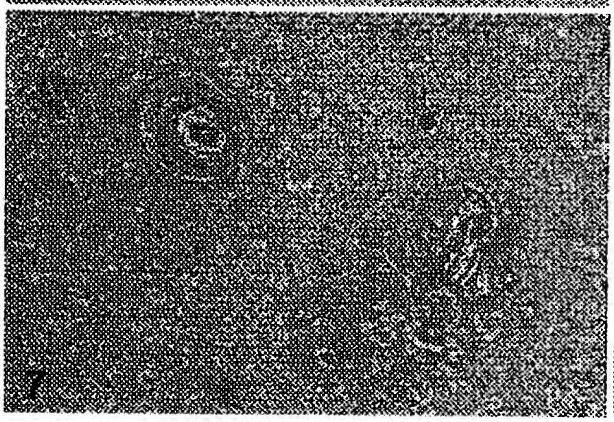
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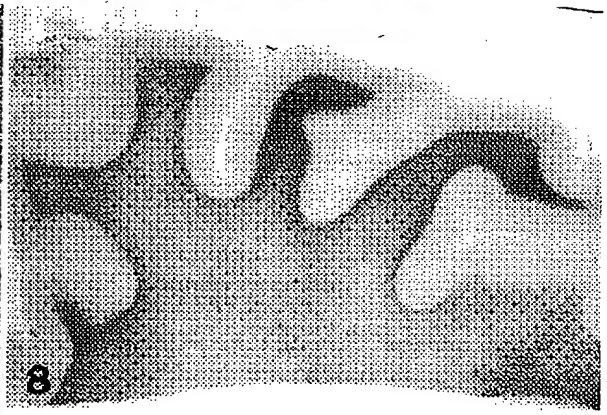
8

Fusahiro Ikuta and Toshiro Kumanishi, and also the nurses at Niigata University Hospital and affiliated hospitals. We also thank Mr. T. Ichikawa, Mr. K. Kobayashi, Mr. T. Hasegawa, and Mrs. C. Tanda for their skillful technical assistance.

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Fig. 1. C7 Segment of the spinal cord from case 3, showing diffuse subarachnoid proliferation, with minimal parenchymal invasion by lymphoma cells, compatible with so-called primary leptomeningeal lymphoma (K-B, ×6)

Fig. 2. Involvement of lymphoma cells in the choroid plexus (H&E, ×75)

Fig. 3. C7 Segment of the spinal cord from case 11, showing massive parenchymal invasion by lymphoma cells (K-B, ×6)

Fig. 4. Spinal cord white matter (Th7 segment) from case 11, showing clusters of tumor cells around the blood vessels (H&E, ×60)

Fig. 5. Involvement of lymphoma cells in the posterior lobe of the pituitary (L26 immunostain, ×8). *A*, Anterior lobe; *P*, posterior lobe

Fig. 6. Pons from case 4 showing necrotic foci (*arrows*) in areas with tumor infiltration (K-B, ×2)

Fig. 7. Right frontal white matter from case 23, showing coagulation necrosis of the invading zone, which contains blood vessels with hyalinous thickening and plasma exudation (H&E, ×90)

Fig. 8. Right parietal lobe from case 23, showing diffuse myelin pallor of the white matter (K-B, ×1.5)

L22 ANSWER 41 OF 112 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1996:563663 BIOSIS
DOCUMENT NUMBER: PREV199799293019
TITLE: The immunologic and endocrinologic status in breast **cancer** patients treated by laser radiation.
AUTHOR(S): Tarutinov, V. I. (1); Gamaleya, N. F.; Sklyar, S. Yu.; Rudykh, Z. M.; Galakhin, K. A.; Skivka, L. M.; Stadnik, V. Ya.; Tkachev, V. A.; Verbetskii, V. V.; Kondrichik, I. G.
CORPORATE SOURCE: (1) Ukr. Res. Inst. Oncol. Radiol., Ukr. Minist. Health, Kiev 252022 Ukraine
SOURCE: Eksperimental'naya Onkologiya, (1996) Vol. 18, No. 3, pp. 240-243.
ISSN: 0204-3564.
DOCUMENT TYPE: Article
LANGUAGE: English
SUMMARY LANGUAGE: English; Russian
AB Intravenous blood irradiation with a low-power helium-neon laser (the wavelength of 633 nm) was undertaken during the pre-operative treatment of 152 breast **cancer** patients (T-2-3, N-1-2, M-0). The course of **laser treatment** induced in 70% of the patients a **tumor** reduction by 20-30%. That allowed to diminish the volume of ionizing radiation therapy. The **laser treatment** resulted in hemopoiesis stimulation and normalization of some immunologic characteristics (blasttransformation activity, natural killer cell activity, rosette-formation by **T cells**, content of circulating **immune** complexes) and also of the endocrinologic status of patients. This status was assessed by determination of six hormones and the activation of pituitary-suprarenal system. In parallel, lowering of the relative estrogenization was established.

L27 ANSWER 84 OF 99 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1992:30161 BIOSIS
DOCUMENT NUMBER: BA93:19436
TITLE: IMMUNOLOGICAL REACTION IN HEPATOCELLULAR **CARCINOMA** BY PEIT EFFECT OF COMBINED USE OF LOCAL INJECTION OF LENTINAN.
AUTHOR(S): YAMAMOTO S; TAKATORI K; OHMOTO K; YAMAMOTO R; IDEGUCHI S; OHUMI T; HINO K; HIRANO Y
CORPORATE SOURCE: DIV. GASTROENTEROLOGY I, DEP. MED., KAWASAKI MED. SCHOOL, 577 MATSUSHIMA, KURASHIKI, OKAYAMA 701-01, JAPAN.
SOURCE: KAWASAKI IGAKKAI SHI, (1989) 15 (3), 429-436.
CODEN: KAIGD3. ISSN: 0386-5924.
FILE SEGMENT: BA; OLD
LANGUAGE: Japanese

AB Fifteen cases of hepatocellular **carcinoma** (HCC) were treated by **percutaneous ethanol** injection therapy (PEIT) and the immunological responses of peripheral blood natural killer (NK) activity and **lymphocyte** subsets were examined. PEIT was done twice, first with ethanol alone, and then with combined use of ethanol and Lentinan. NK activity and **lymphocyte** subsets were examined three times; before, one day and seven days after PEIT. NK activity was significantly decreased from 26.1 .+-. 13.0% to 15.9 .+-. 11.1% on the day following PEIT. With combined use of ethanol and Lentinan, NK activity was 21.5 .+-. 14.4% before injection, 21.8 .+-. 16.9% on the following day, and 30.3 .+-. 14.5% seven days after injection. Regarding **lymphocyte** subsets OKT 4 increased significantly after combined use of ethanol and Lentinan, while no apparent changes were noted following ethanol injection alone. From the above results, it was concluded that Lentinan injection may be useful in the enhancement of the systemic immunological reaction against HCC.

L27 ANSWER 83 OF 99 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1992:30162 BIOSIS
DOCUMENT NUMBER: BA93:19437
TITLE: **PERCUTANEOUS ETHANOL** INJECTION THERAPY PEIT EXPERIMENTAL AND CLINICAL STUDIES.
AUTHOR(S): YAMAMOTO S; TAKATORI K; OHMOTO K; IDEGUCHI S; YAMAMOTO R; OHUMI T; HINO K; HIRANO Y
CORPORATE SOURCE: DIV. GASTROENTEROLOGY I, DEP. MED. KAWASAKI MED. SCHOOL, 577 MATSUSHIMA, KURASHIKI, OKAYAMA 701-01, JAPAN.
SOURCE: KAWASAKI IGAKKAI SHI, (1989) 15 (3), 437-444.
CODEN: KAIGD3. ISSN: 0386-5924.
FILE SEGMENT: BA; OLD
LANGUAGE: Japanese

AB The usefulness of **percutaneous ethanol** injection therapy (PEIT) under ultrasonic guidance was examined in both experimental and clinical liver **cancer**, and its influence on the host **immune** response was also investigated. Natural killer (NK) activity, **NK cell** surface markers and **T cell** subsets were compared in PEIT and intratumoral injection of Lentinan and OK-432. 1. Pathological examination of DAB-induced rat liver **cancer** revealed that ethanol induced complete necrosis as early as 2 days after injection, but the area of necrosis was localized and limited in extent. 2. Decrease in NK activity, increase in the OKT 4 and 4/8 ratio and decrease in Leu7- CD16+ were noted in PEIT. 3. Intratumoral injection of Lentinan and OK-432 induced increases in NK activity and OKT 8. 4. OK-432 increased Leu7- CD16+ and combined injection of ethanol and OK-432 prevented decrease in Leu7- CD16+.

L27 ANSWER 79 OF 99 MEDLINE DUPLICATE 23
ACCESSION NUMBER: 91096249 MEDLINE
DOCUMENT NUMBER: 91096249 PubMed ID: 2267690
TITLE: [Adoptive **laser immunotherapy** and photodynamic **therapy** in ORL oncology]. Adoptivnaia lazernaia immunoterapiia i fotodinamicheskaia terapiia v LOR-onkologii.
AUTHOR: Antoniv V F; Dmitriev A A; Daikhes N A; Ivanov A V; Davudov Kh Sh; Perekosova Iu V; Laptev V P
SOURCE: VESTNIK OTORINOLARINGOLOGII, **(1990 Sep-Oct)** (5) 3-8.
Journal code: 0416577. ISSN: 0042-4668.
PUB. COUNTRY: USSR
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: Russian
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199102
ENTRY DATE: Entered STN: 19910322
Last Updated on STN: 19970203
Entered Medline: 19910208

AB Present-day developments in oncological applications of **laser therapy** are adaptive **laser immunotherapy** (ALIT) and photodynamic therapy (PDT). ALIT (helium-neon laser) was used in 35 ENT-**cancer** patients to irradiate immunocompetent leukocytes isolated from blood in an Amino cell separator. The use of ALIT in the combined treatment of our patients improved their health condition.

Cytological, immunochemical and immunological examinations of blood revealed an increased count of activated **lymphocytes**, normalization of acute-phase proteins, stimulation of cell-mediated immunity and nonspecific resistance. The pharmacokinetics and photodynamic activity of porphyrin compounds in mice with inoculated **tumors** were investigated. Experimental observations demonstrated that the application of photodynamic therapy of hematoporphyrins in ENT-oncology seems very promising.

L22 ANSWER 13 OF 112 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1999:449676 CAPLUS
DOCUMENT NUMBER: 132:90121
TITLE: Photothermal and immunological reactions against metastatic **tumors** using laser photosensitizer immunoadjuvant
AUTHOR(S): Chen, Wei R.; El-Samad, Ahmad; Nordquist, Robert E.
CORPORATE SOURCE: Oklahoma School of Science and Mathematics, Oklahoma City, OK, USA
SOURCE: Proceedings of SPIE-The International Society for Optical Engineering (**1999**), 3601(Laser-Tissue Interaction X: Photochemical, Photothermal, and Photomechanical), 75-81
CODEN: PSISDG; ISSN: 0277-786X
PUBLISHER: SPIE-The International Society for Optical Engineering
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Photothermal tissue interaction is the most common phenomenon when laser energy is deposited in tissue. Because of the sensitivity of **cancer** cells to temp. increase, photothermal reaction can be an effective mechanism of direct **cancer** destruction using lasers. **Tumor**-specific **immune** response is crucial in achieving systemic and long-term cures for **cancers**, particularly for metastatic **cancers**. Can photothermal interaction induce sufficient immunol. reaction when the local destruction of **tumor** cells occurs To achieve selective photothermal destruction, indocyanine green as a photosensitizer was directly injected into rat mammary **tumors**, followed by irradn. of 805 nm laser light. Although extensive photothermal **tumor** killing was achieved and **tumor** growth was slowed down immediately following the treatment, photothermal reaction alone was shown not sufficient in controlling the treated primary **tumors** and their metastases. When an immunoadjuvant was used with the indocyanine green, however, the same **laser treatment** not only could eventually eradicate the treated primary **tumors** but also eradicate the untreated metastases at remote sites. The **tumor** eradication went through a growth-regression process over a period of six to nine weeks post-treatment, indicating an induced **immune** response. The Western Blot anal. using the serum from a **laser-immunotherapy** cured rat showed that the **tumor**-specific antibody induced by the treatment had a long- lasting effect. Our exptl. data indicated that photothermal interaction alone was not sufficient to slow and eventually reverse **tumor** growth. However, it can reduce the **tumor** burden and at the same time release **tumor** antigens to be recognized by the host **immune** system. Therefore, in conjunction with specific immunol. stimulation using in situ immunoadjuvants, the selective thermal injury to **tumors** plays an important and a direct role in this **laser immunotherapy**.

REFERENCE COUNT: 14 THERE ARE 14 CITED REFERENCES AVAILABLE FOR THIS

L22 ANSWER 12 OF 112 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1999:449677 CAPLUS
DOCUMENT NUMBER: 132:90122
TITLE: Photodynamic therapy and **immune** response in **tumor**-bearing mice
AUTHOR(S): Canti, Gianfranco L.; Cubeddu, Rinaldo; Taroni, Paola; Valentini, Gianluca
CORPORATE SOURCE: Dep. Pharmacol., Sch. Med., Univ. of Milan, Milan, Italy
SOURCE: Proceedings of SPIE-The International Society for Optical Engineering (**1999**), 3601(Laser-Tissue Interaction X: Photochemical, Photothermal, and Photomechanical), 82-88
CODEN: PSISDG; ISSN: 0277-786X
PUBLISHER: SPIE-The International Society for Optical Engineering
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Since **immune** response of the host is important in the control of **tumor** growth and spreading, and the Photodynamic therapy (PDT) is able to increase the antitumor immunity, in our lab. we examine the effect of PDT on **immune** compartment of **tumor** bearing mice. **Lymphocytes** and **macrophages** collected from **tumor** bearing mice pretreated with PDT are cytotoxic in vitro and in vivo against the parental **tumor** lines, in contrast the same **immune** cells population collected from **tumor** bearing mice pretreated only with laser light are unable to lyse the parental **tumor** cells. In adoptive immunotherapy expts., treatment of mice bearing MS-2 **tumor** with adoptive transfer of **immune** **lymphocytes** collected from mice pretreated with PDT is able to significantly increase the survival time; in contrast the **lymphocytes** collected from mice pretreated only with laser light were not able to modify the survival time suggesting that the **laser treatment** alone did not increase the **immune** response of the host. In conclusion these results demonstrate that the PDT induce a strong **immune** response on the host and the stimulated **lymphocytes** generated could be used for an adoptive immunotherapy approach; moreover **laser** **treatment** alone (thermal effect) is unable to modulate the **immune** response of the host.
REFERENCE COUNT: 20 THERE ARE 20 CITED REFERENCES AVAILABLE FOR THIS

L22 ANSWER 8 OF 112 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 1999393277 MEDLINE
DOCUMENT NUMBER: 99393277 PubMed ID: 10465586
TITLE: Effects of transpupillary thermotherapy on immunological parameters and apoptosis in a case of primary uveal melanoma.
AUTHOR: Schurmans L R; Blom D J; De Waard-Siebinga I; Keunen J E; Prause J U; Jager M J
CORPORATE SOURCE: The Department of Ophthalmology, Leiden University Medical Center, The Netherlands.. schurmans@worldonline.nl
SOURCE: MELANOMA RESEARCH, **(1999 Jun)** 9 (3) 297-302.
Journal code: 9109623. ISSN: 0960-8931.
PUB. COUNTRY: ENGLAND: United Kingdom
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199911
ENTRY DATE: Entered STN: 20000111
Last Updated on STN: 20000111
Entered Medline: 19991103

AB Transpupillary thermotherapy (TTT) is a new treatment modality for uveal melanoma. We studied whether application of TTT influences the immunogenicity of the **tumour** cells in vivo or the expression of molecules related to apoptosis. Immunohistochemistry using monoclonal antibodies directed against HLA molecules, HMB45, P53, Fas ligand (FasL), Fas, Bcl-2 and **tumour**-infiltrating cells was applied to sections of an enucleated eye containing a uveal melanoma that received TTT 1 week before enucleation. The innermost part of the **tumour** which had been exposed directly to the **laser treatment** showed no staining for HLA antigens, nor for Fas or FasL epitopes. The intermediate part of the **tumour** showed a wet necrosis and HLA expression similar to the expression in the peripheral **tumour**. A large number of **macrophages** were observed in the necrotic as well as the intact **tumour** tissue, especially bordering the wet necrotic area. FasL and Bcl-2 were only expressed in the viable, outer part of the **tumour**. This immunological evaluation of one case of uveal melanoma treated with TTT revealed that TTT may not only have a direct destructive effect on the primary **tumour**, but may also influence the immunogenicity of uveal melanoma cells, induce infiltration of **macrophages** into the **tumour**, and induce apoptosis. The presence of many **macrophages** suggests that they play a role in the removal of the TTT-treated **tumour** tissue by phagocytosis.

L22 ANSWER 3 OF 112 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1999:261946 BIOSIS
DOCUMENT NUMBER: PREV199900261946
TITLE: Long-term **tumor** resistance induced by **laser** photo-**immunotherapy**.
AUTHOR(S): Chen, Wei R. (1); Zhu, Wei-Guo; Dynlacht, Joseph R.; Liu, Hong; Nordquist, Robert E.
CORPORATE SOURCE: (1) Department of Physics, Oklahoma School of Science and Mathematics, 1141 N. Lincoln Blvd., Oklahoma City, OK, 73104 USA
SOURCE: International Journal of Cancer, (**May 31, 1999**) Vol. 81, No. 5, pp. 808-812.
ISSN: 0020-7136.
DOCUMENT TYPE: Article
LANGUAGE: English
SUMMARY LANGUAGE: English
AB An ideal treatment modality for metastasizing **tumors** should eradicate the primary **tumor** and elicit a systemic, **tumor**-selective response leading to elimination of metastases and long-term **tumor** resistance. Also, it should be induced by local treatment at the primary site, to limit adverse systemic effects. A new method for treating metastatic **tumors** which utilizes a combination of a near-infrared laser, a photosensitizer and an immunoadjuvant has been developed. It involves intra-**tumor** injection of the sensitizer/adjuvant solution, followed by local non-invasive laser irradiation. It has produced regression and total eradication of treated primary **tumors** and untreated metastases at remote sites against mammary **tumors** in rats. Successfully treated **tumor**-bearing rats showed total **tumor** resistance to subsequent **tumor** rechallenge. Our histochemical results showed that sera from cured **tumor**-bearing rats contained antibodies that bound strongly to the plasma membrane of both living and preserved **tumor** cells. Western blot analysis of **tumor** cell proteins using sera from successfully treated rats as the source of primary antibodies also showed distinct bands, indicating induction of **tumor**-selective antibodies. Our findings indicate that a systemic, long-term effect on metastatic **tumors** can be induced by local application of **laser** photo-**immunotherapy**.

L27 ANSWER 91 OF 99 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1988:92935 BIOSIS
DOCUMENT NUMBER: BA85:49707
TITLE: CLINICAL **LASER** PHOTODYNAMIC **THERAPY** IN THE TREATMENT OF BLADDER **CARCINOMA**.
AUTHOR(S): SHUMAKER B P; HETZEL F W
CORPORATE SOURCE: DEP. UROL., HENRY FORD HOSP., NORTH WOODWARD UROL. ASSOCIATES, PONTIAC, MICH. 48053.
SOURCE: PHOTOCHEM PHOTOBIOL, (1987) 46 (5), 899-902. CODEN: PHCBAP. ISSN: 0031-8655.
FILE SEGMENT: BA; OLD
LANGUAGE: English

AB The treatment of bladder **carcinoma** using dihematoporphyrin ether (DHE) and **laser** photodynamic **therapy** (PDT) is described herein. Patients selected for this study have cytology- and biopsy-proven transitional cell **carcinoma**, no histologic evidence of muscle invasion, and negative excretory urograms. Sixteen patients have been treated, with follow-up from 6 to 36 months. Eleven have had a complete response, and 3 a partial response in that they required re-treatment for recurrence. Two of these patients have not recurred at this time. One of the patients who recurred had **tumor** extension into the prostatic urethra and has been successfully re-treated (disease-free at 6 months). Three was one treatment failure and 1 patient lost to follow-up. Photosensitivity for up to 4 weeks is a known side-effect, but unexpected morbidity included a transient but significant increase in urinary frequency, urgency, and occasionally hematuria which spontaneously resolved within 3-4 weeks. Careful placement of the fiberoptic tip of the centre of the bladder, bladder distension during treatment with saline rather than water, the instillation of the minimum volume required to "smooth out" the mucosa for complete bladder photoradiation, and delivered energy of 25 J cm-1 or less may have prevented the more severe complications (i.e. bladder shrinkage) reported by Dougherty and Nseyo (personal communication). We also feel that there is some early evidence that a heightened **immune** response (similar to intravesical BCG) may potentially play some role in explaining the efficacy of PDT in a long disease-free intervals, although this is just a histologic observation at present. It appears the PDT offers another practical treatment modality for non-invasive transitional cell **carcinoma** in patients refractory to standard surgical and chemotherapeutic regimens, and has been addressed by numerous other investigators such as Benson (1985) and Hisazumi (1983). We are presently recommending to our patients in these categories to undergo a course of PDT prior to relinquishing to cystectomy.

L27 ANSWER 92 OF 99 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

L27 ANSWER 82 OF 99 MEDLINE DUPLICATE 26
ACCESSION NUMBER: 89331633 MEDLINE
DOCUMENT NUMBER: 89331633 PubMed ID: 2755999
TITLE: Effect of photodynamic therapy on anti-**tumor immune** defenses: comparison of the photosensitizers hematoporphyrin derivative and chloro-aluminum sulfonated phthalocyanine.
AUTHOR: Marshall J F; Chan W S; Hart I R
SOURCE: PHOTOCHEMISTRY AND PHOTOBIOLOGY, **(1989 May)** 49 (5) 627-32.
Journal code: 0376425. ISSN: 0031-8655.
PUB. COUNTRY: ENGLAND: United Kingdom
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198909
ENTRY DATE: Entered STN: 19900309
Last Updated on STN: 19900309
Entered Medline: 19890907

AB The effects of the two photosensitizers chloroaluminum sulfonated phthalocyanine (ClAlSPc) and hematoporphyrin derivative (HpD) on the functional activities of **macrophages** and natural killer (**NK**) **cells,** two immunocyte populations implicated in the control of **tumor** development and spread, have been investigated. Murine peritoneal **macrophages** treated in vivo with ClAlSPc or HpD at 10 mg/kg body weight showed no impairment of Fc-mediated phagocytic capacity and only minor disturbances of in vitro tumoricidal/tumoristatic function. The **NK cell** activity of splenocytes obtained from photosensitizer-treated mice, assayed 24 or 48 h after i.v. injection of ClAlSPc or HpD at 10 mg/kg was unaffected compared to controls. However significant inhibition of NK activity was observed when splenocytes obtained from mice with or without subcutaneous Colo 26 **tumors,** treated with ClAlSPc plus **laser therapy** (675 nm) were used as effector cells. The results show that impairment of some anti-**tumor** activity can be observed in phthalocyanine treated or phthalocyanine + laser-treated animals but this relatively minor impairment may augur well for the use of systemic phthalocyanine administration in photodynamic therapy.

L27 ANSWER 78 OF 99 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1991:320749 BIOSIS
DOCUMENT NUMBER: BA92:31264
TITLE: RESPONSE OF MURINE MAMMARY **ADENOCARCINOMA** TO PHOTODYNAMIC THERAPY AND IMMUNOTHERAPY.
AUTHOR(S): DIMA F V; VASILIU V; MIHAILESCU I N; DIMA V S; POPA A; STIRBET M
CORPORATE SOURCE: CANTACUZINO-INST., DEP. EXP. PATHOL., BUCHAREST, ROMANIA.
SOURCE: LASER THER, (1990) 2 (4), 153-160.
CODEN: LATHE5.
FILE SEGMENT: BA; OLD
LANGUAGE: English

AB A murine mammary adenocarcinome was treated with intraperitoneal photofrin
II and laser (10 Mw, 632 nm, He-Ne laser). Photofrin II was given intraperitoneally 4 h before 30-min **laser treatment**. A total of six photofrin II/**laser treatments**, given at 3-4 day intervals, resulted in 52.6% complete response, the cure rate was 26.3%; BCT or PD60 were given in association with PDT to CBA mice with mammary **carcinoma**. Addition of nonspecific immunostimulants significantly increased the antitumour effects of PDT, and the combination of PDT + PD60 resulted in the highest incidence of complete **tumour** regression (82.1%). Enhanced activity of phytohemagglutinin-M response T-**lymphocytes** and natural killer cells capable of killing K562 **tumour** cells was noted. These experimental observations i.e. marked inhibition of the mammary **adenocarcinoma** developed due the 'combined' PDT-immunotherapy, suggest the possible use of this new methodology as an adjuvant in clinical practice.

L27 ANSWER 74 OF 99 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1992:237306 BIOSIS
DOCUMENT NUMBER: BA93:125331
TITLE: PREOPERATIVE ACTIVATION OF THE **IMMUNE** SYSTEM BY LOW REACTIVE LEVEL **LASER THERAPY** LLLT IN ONCOLOGIC PATIENTS A PRELIMINARY REPORT.
AUTHOR(S): SKOBELKIN O K; MICHAILOV V A; ZAKHAROV S D
CORPORATE SOURCE: INST. LASER MED., 123242 MOSCOW, RUSS.
SOURCE: LASER THER, (1991) 3 (4), 169-175.
CODEN: LATHE5.
FILE SEGMENT: BA; OLD
LANGUAGE: English

AB In vitro cellular and in vivo animal studies have pointed to the possible boosting effect of Low reactive-Level **Laser Therapy** (LLLT) on the autoimmune system of immunodeficient **cancer** -inoculated animals, resulting in an increase in the expected life-span of the irradiated animals. Following such studies, the authors designed a study to evaluate the effect of LLLT as an adjunctive therapy for conventional surgical intervention in **cancer** in man. A comparative study of different types of irradiation from low incident energy level lasers were performed on 60 oncologic patients, irradiation being delivered during the immediate preoperative period. External irradiation with a semiconductor laser (wavelength 890 nm); internal irradiation with a helium-neon laser (wavelength 632.8 nm); and a combination of both methods was applied. The most effective irradiation was the external one made with a semiconductor laser. Studies were carried out on white cell components in blood, assays of immunoglobulin activity (IgA, IgM and IgG) were made, in addition to the determination of the behaviour of T-**lymphocyte** fractions (active roseate **T-cells**, T-helpers and T-suppressors) post LLLT. It was seen from the data that the total immunoresponse actually increased following LLLT, with no visible increase in tumoural remnant size. Although more detailed qualitative experimental and controlled work must be done before ths application of LLLT can be carried out on a regular basis, the authors feel strongly that in this preliminary report, the findings point to an exciting and possible use for LLLT, in particular for the photoactivation of the autoimmune system and tumoural antigen photomodification, and in general for the treatment of immunodeficiency.

L27 ANSWER 72 OF 99 MEDLINE DUPLICATE 20
ACCESSION NUMBER: 93009090 MEDLINE
DOCUMENT NUMBER: 93009090 PubMed ID: 1395099
TITLE: Evaluation of serum levels of **tumour** necrosis factor-alpha (TNF-alpha) and soluble IL-2 receptor (sIL-2R)
and CD4, CD8 and natural killer (NK) populations during infrared pulsed **laser** device (IPLD) **treatment**.
AUTHOR: Santana-Blank L A; Castes M; Rojas M E; Vargas F; Scott-Algara D
CORPORATE SOURCE: FUNDALAS, Centro Clinico Profesional del Oeste, El Paraiso-Caracas, Venezuela.
SOURCE: CLINICAL AND EXPERIMENTAL IMMUNOLOGY, **(1992 Oct)** 90 (1) 43-8.
Journal code: 0057202. ISSN: 0009-9104.
PUB. COUNTRY: ENGLAND: United Kingdom
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199211
ENTRY DATE: Entered STN: 19930122
Last Updated on STN: 19930122
Entered Medline: 19921105

AB The purpose of this study was to evaluate serum levels of TNF-alpha, sIL-2R and distribution of peripheral leucocyte subsets in patients with advanced **neoplastic** disease undergoing IPLD treatment. Fifteen **cancer** patients with evidence of persistent disease were further divided in two groups according to outcome at the end of the period of clinical evaluation: group 1 patients were still alive and group 2 patients had died. Our results show: (i) an increase in the initial level of TNF-alpha in both groups; (ii) a decrease in TNF-alpha levels during the follow up of group 1 patients; (iii) a significant increase in serum levels of sIL-2R in patients in group 2 compared with those in group 1; (iv) a progressive and constant increase in TNF-alpha levels in group 2; (v) a decrease in CD4+CD45RA+ subpopulation in both groups; (vi) an increase in CD25+ cells; (vii) an increase in CD4+, CD4+CD45RA+ and CD25+ cells during the follow up of group 2 patients. The data generated here form the basis for further investigations on the use of IPLD as a single agent and in combination with other biological response modifiers in **cancer** patients.

L27 ANSWER 70 OF 99 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1992:392040 BIOSIS
DOCUMENT NUMBER: BA94:64215
TITLE: AUGMENTATION OF THE SYSTEMIC HOST ANTI-**TUMOR** RESPONSE THROUGH LASER EXCISION.
AUTHOR(S): BRIEN T P; LANZAFAME R J; NAIM J O; ROGERS D W; WANG M J; HINSHAW J R
CORPORATE SOURCE: 1445 PORTLAND AVE. G06, ROCHESTER, N.Y. 14621.
SOURCE: LASERS SURG MED, (1992) 12 (3), 313-317.
CODEN: LSMEDI. ISSN: 0196-8092.
FILE SEGMENT: BA; OLD
LANGUAGE: English

AB This study examines whether primary laser excision results in augmentation of the systemic host anti-**tumor** response to **tumor** rechallenge. Single R3230AC mammary **tumor** implants, (0.5 .times. 0.5 .times. 1.0 mm), were grown in 112 female Fisher 344 rats. The animals were randomized. Group S **tumors** were excised by scalpel. Group E was excised with a Surgistat electrocautery (Valley Labs, Boulder, CO). Group CS was excised with a Sharplan 1100 CO2 laser (Sharplan, Allendale, NJ) at 25 watts (W) continuous wave (CW) (0.2 mm spot size) and the wound was "sterilized" with a 5-mm spot size by gently heating the tissue without blanching. Group K was excised with a KTP/532 laser (Laserscope, San Jose, CA) at 17 W CW using a 400 .mu.m fiber. Group Y was excised with a Sharplan 2100 Nd:YAG laser set at 15 W CW using a 0.2 mm clear sapphire tip. A second **tumor** implant (0.5 .times. 0.5 .times. 1.0 mm), was placed at a remote site 14 days postoperatively. An unoperated control group was implanted. Secondary **tumor** volumes were measured for 36 days and the mean **tumor** volumes (MTV) were statistically compared. The MTV in groups CS, K, Y, and E was less than control ($P < 0.01$). The MTV in groups CS, K, Y, and E was less than group S, although this was not statistically different. Lasers and cautery appear to increase the host response against subsequent **tumor** challenge. This study corroborates earlier studies of other modalities. Further studies to determine whether this host sensitization is an **immune** response and to elucidate the mechanisms of this effect are warranted.

L27 ANSWER 69 OF 99 MEDLINE DUPLICATE 18
ACCESSION NUMBER: 93043959 MEDLINE
DOCUMENT NUMBER: 93043959 PubMed ID: 1330113
TITLE: Indication of liver transplantation for hepatocellular **carcinoma** in Japan.
AUTHOR: Tanikawa K
CORPORATE SOURCE: Second Department of Medicine, Kurume University School of Medicine, Japan.
SOURCE: SURGERY TODAY, **(1992)** 22 (5) 395-400. Ref: 7
Journal code: 9204360. ISSN: 0941-1291.
PUB. COUNTRY: Japan
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199212
ENTRY DATE: Entered STN: 19930122
Last Updated on STN: 19980206
Entered Medline: 19921204

AB Approximately 20,000 patients die of hepatocellular **carcinoma** (HCC) annually in Japan and most of them are hepatitis B virus (HBV) or hepatitis C virus (HCV) carriers. Recently, small HCC, less than 3 cm in diameter, have frequently been found by ultrasonography in the follow-up of patients with chronic liver diseases. Such cases are mainly treated by either surgical resection or **percutaneous ethanol** injection therapy (PEIT) with a satisfactory 5 year survival rate of 50%. In addition, the survival rate of advanced cases has gradually improved thanks to transcatheter arterial chemo-embolization combined with PEIT, radiation, hyperthermia, or **immune** therapy. On the other hand, our autopsy study has indicated a high frequency of extrahepatic metastasis in advanced cases. From these results, liver transplantation for HCC does not seem to be the treatment of first choice, at present, in Japan. In the future, the means to control the underlying infection of HBV or HCV as well as making an accurate imaging diagnosis for the detection of extrahepatic metastasis will become inevitably more important for successful liver transplantation in HCC.

L27 ANSWER 67 OF 99 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1994:79548 BIOSIS
DOCUMENT NUMBER: PREV199497092548
TITLE: Studies on some biological functions of **macrophages** activated by HeNe **laser** photodynamic **treatment** as compared to Corynebacterium parvum and interferon activation.
AUTHOR(S): Dima, F. Vasile (1); Vasiliu, V.; Ionescu, M. D.; Dima, S. V.
CORPORATE SOURCE: (1) Cantacuzino Inst., CP 1-525, R 70 100, Bucharest Romania
SOURCE: Laser Therapy, (1993) Vol. 5, No. 3, pp. 117-124. ISSN: 0898-5901.
DOCUMENT TYPE: Article
LANGUAGE: English
AB Some biological properties of **macrophages** activated with Corynebacterium parvum, recombinant murine interferon-gamma (rMuIFN-gamma) and HeNe **laser** photodynamic **therapy** (PDT) were studied. Results obtained indicate the following: (i) **macrophages** activated with the three immunopotentiators have an intense phagocytic activity; (ii) the association of PDT-treated T and B peritoneal **lymphocytes** with **macrophages** increases their biological properties; (iii) the cytotoxic activity of photodynamically activated **macrophages** (PD - AM-vphi) varied between 42.8 and 59.4% of the normal function of the **tumour** target cells; and (iv) the three sets of **macrophages** exert cytostatic effects upon human and mouse leukaemia cells. In conclusion, **macrophage** immunopotentiation with photodynamic therapy has proved to be a useful method in cellular biological studies as well as for adoptive immunotherapy in various **cancer** forms.

L22 ANSWER 65 OF 112 MEDLINE DUPLICATE 20
ACCESSION NUMBER: 94172898 MEDLINE
DOCUMENT NUMBER: 94172898 PubMed ID: 8126975
TITLE: Possible application of the laser in immunobiology.
AUTHOR: Tadakuma T
CORPORATE SOURCE: Department of Microbiology, School of Medicine, Keio University, Tokyo, Japan.
SOURCE: KEIO JOURNAL OF MEDICINE, **(1993 Dec)** 42 (4) 180-2. Ref: 4
Journal code: 0376354. ISSN: 0022-9717.
PUB. COUNTRY: Japan
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW LITERATURE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199404
ENTRY DATE: Entered STN: 19940420
Last Updated on STN: 19940420
Entered Medline: 19940408

AB The human **immune** system acts a defence mechanism against exogenous or indigenous potentially harmful bodies, such as bacteria and viruses. The major histocompatibility complex (MHC class I and class II antigens) form key elements of legitimate body components, and the organization of MHC molecules allows T-**lymphocytes** to distinguish between legitimate and foreign bodies. On detection of a foreign component, **T-cells** activate the necessary pathways for destruction of the foreign body. Occasionally however the system breaks down and the result is a disease of an autoimmune nature. Both visible light and infrared low reactive-level **laser therapy** (LLLT) has been shown to act on **immune** system cells in a number of ways, activating the irradiated cells to a higher level of activity. Infrared LLLT has been shown to increase both the phagocytic and chemotactic activity of human leukocytes in vitro, for example. This is an example of photobiological activation. Photobiological cell-specific destruction is also possible using doses of low incident laser energy on cells which have been photosensitized for the specific wavelength of the laser, such as in photodynamic therapy (PDT) for superficial **cancers**. LLLT has also been shown to act directly and selectively on the autoimmune system, restoring immunocompetence to immunocompetence cells. Although much more research needs to be done, there are enough experimental and clinical data to show that the laser, and LLLT in particular, has a possibly exciting role both in immunobiological therapy for diseases of the **immune** system, and to activate and boost the normal reaction of the **immune** system components against harmful foreign bodies.

L22 ANSWER 60 OF 112 MEDLINE DUPLICATE 18

ACCESSION NUMBER: 94185237 MEDLINE
DOCUMENT NUMBER: 94185237 PubMed ID: 8137477
TITLE: Clinical study of biological response modifiers as maintenance therapy for hepatocellular **carcinoma**.
AUTHOR: Suto T; Fukuda S; Moriya N; Watanabe Y; Sasaki D; Yoshida Y; Sakata Y
CORPORATE SOURCE: First Department of Internal Medicine, Hirosaki University School of Medicine, Japan.
SOURCE: CANCER CHEMOTHERAPY AND PHARMACOLOGY, **(1994)** 33 Suppl S145-8.
Journal code: 7806519. ISSN: 0344-5704.
PUB. COUNTRY: GERMANY: Germany, Federal Republic of
DOCUMENT TYPE: (CLINICAL TRIAL)
Journal; Article; (JOURNAL ARTICLE)
(RANDOMIZED CONTROLLED TRIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199404
ENTRY DATE: Entered STN: 19940509
Last Updated on STN: 19980206
Entered Medline: 19940426

AB We conducted a randomized, controlled trial comparing 5-fluorouracil (5-FU) with or without biological response modifiers (BRMs) as a maintenance therapy for hepatocellular **carcinoma** (HCC) after treatment with **percutaneous ethanol** injection (PEI), transcatheter arterial embolization (TAE) or arterial infusion of antitumor agents (AI). A total of 58 cases of HCC were classified into 4 groups as follows: group I, PSK with 5-FU (n = 15); group II, lentinan with 5-FU (n = 15); group III, OK-432 with 5-FU (n = 12); and group IV, 5-FU alone as the control (n = 16). The mean survival time, mortality rate, time to progression, and T4/T8 ratio of **lymphocytes** in the peripheral blood were compared among the four groups. There was no significant difference in the background factors among the groups. In group I, the T4/T8 ratio of **lymphocytes** was reduced after the therapy. No significant difference was found among the groups in terms of the mean survival time, mortality rate, or time to progression. PEI for initial therapy was superior to the other therapies in terms of the mean survival time and mortality rate. These results suggest that the addition of BRM to maintenance therapy with 5-FU exerts no prognostic benefit on HCC patients treated with PEI, TAE, or AI.

L9 ANSWER 8 OF 12 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1992:259964 BIOSIS
DOCUMENT NUMBER: BA93:136289
TITLE: DOSE-RELATED IMMUNOLOGICAL AND MORPHOLOGICAL CHANGES OBSERVED IN RATS WITH WALKER-256 CARCINOSARCOMA AFTER PHOTODYNAMIC THERAPY A CONTROLLED STUDY.
AUTHOR(S): **DIMA F V**; VASILIU V; MIHAILESCU I N; **DIMA S V**; STIRBET M; POPA A; LACKY D
CORPORATE SOURCE: INSTITUT CANTACUZINA, CP 1-525, R 70.100 BUCHAREST, ROMANIA.
SOURCE: LASER THER, (1991) 3 (4), 159-168.
CODEN: LATHE5.
FILE SEGMENT: BA; OLD
LANGUAGE: English

AB Experiments were performed on six batches of Wistar inbred rats with Walker-256 carcinosarcoma 7 days post-transplantation. Animals from batches I and II were exposed to photofrin II (20 mg/kg body mass) or HeNe **laser** (10 mW; 632.8 nm), respectively; the animals in the batches III-V were given photofrin II, intraperitoneally, 24 h before 60-min **laser** treatment: one, three and six photofrin/**laser** treatments, respectively, were applied at an interval of 3 days. The control batch (batch VI) consisted of animals presenting with untreated Walker-256 tumours. The results were as follows: photofrin II or HeNe **laser** alone (photoexposure to low doses of 15 J/cm2) had no significant effects on tumoural volume and the survival of the rats. Photoexposure to multiple doses of PDT led to complete regression of tumoural volume (65.8%); the cure rate was 31.5% and concomitantly survival rates increased. Cell-mediated immunity tests (performed at 7 and 28 days post-treatment) underlined superior values in batch IV and V animals photoexposed to multiple PDT doses, in comparison with immuno-suppression noticed in batches I-III and the control batch VI. Data presented in this work demonstrate that photodynamic treatment exposure using multiple doses stimulates cell-mediated antitumoural activity, induces modifications in tumoural histological structure, increases survival rates and reduces tumoural incidence in Walker-256 carcinosarcoma in the rat model.

L22 ANSWER 112 OF 112 CANCERLIT
ACCESSION NUMBER: 77804333 CANCERLIT
DOCUMENT NUMBER: 77804333
TITLE: MICROWAVES, MAGNETIC IRON PARTICLES AND LASERS AS A COMBINED TEST MODEL FOR INVESTIGATION OF HYPERTHERMIA TREATMENT OF **CANCER**.
AUTHOR: Goldman L; Dreffer R
CORPORATE SOURCE: Laser Lab., Dept. Dermatology Medical Center, Cincinnati, OH.
SOURCE: Arch Dermatol, **(1976)** 257 (2) 227-232.
ISSN: 0003-987X.
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Hierarchical Classification of Proteins
ENTRY MONTH: 197706
ENTRY DATE: Entered STN: 19941107
Last Updated on STN: 19941107

AB Various adjuvants in selective local hyperthermia were studied. Microcrystalline iron particles, ferrofluids, or Imferon B (a complex of ferric dioxide and dextran) were injected into small pockets in the abdominal skin of eight rabbits and temperature sensors introduced. Biopsies from the iron depot areas and adjacent skin showed diffuse spread of iron particles with globular masses and intracellular iron particles with iron stains. There was some **lymphocyte** infiltration and an intense histiocytic response. Deep thermal **coagulation necrosis** occurred after all laser impacts, which were delivered into the open pocket area. A superficial depot of iron particles induced local tissue hyperthermia of 15-20 degrees during microwave exposure. No direct temperature measurements were made with laser exposure, but little heat transmission to adjacent tissue was found in earlier experiments on dogs. (17 refs)

L22 ANSWER 45 OF 112 MEDLINE DUPLICATE 14
ACCESSION NUMBER: 96270970 MEDLINE
DOCUMENT NUMBER: 96270970 PubMed ID: 8925597
TITLE: A clinicopathological study of **malignant** glioma done after local administration of chemotherapeutic agents.
AUTHOR: Shimura T; Teramoto A; Nakazawa S; Aihara K
CORPORATE SOURCE: Department of Neurosurgery, Nippon Medical School, Tokyo, Japan.
SOURCE: CLINICAL NEUROPATHOLOGY, **(1996 Mar-Apr)** 15 (2) 119-24.
Journal code: 8214420. ISSN: 0722-5091.
PUB. COUNTRY: GERMANY: Germany, Federal Republic of
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199611
ENTRY DATE: Entered STN: 19961219
Last Updated on STN: 19961219
Entered Medline: 19961118

AB Light and electron microscopic studies on **tumor** tissue from 5 autopsied and 7 rebiopsied patients with **malignant** glioma after receiving intratumor administration of anti-**neoplastic** agents were made. Four patients were correlated with their serial MRI. After craniotomy 0.5 mg of adriamycin was administered using an Ommaya reservoir into the **tumor** bed. Light microscopy of the recurrent **tumor** and adjacent necrotic tissue revealed massive **coagulation necrosis** which was aspirated into the tip of the Ommaya tube. Around the massive **coagulation necrosis** and cystic cavity, abundant reactive collagenous tissues, gliomesenchymal tissue, infiltrating **lymphocytes**, and a small amount of foreign body giant cells were found concomitantly with organized necrotic tissue. The electron microscopic study of the above mentioned tissue showed deposits of lipofuscin, lipid droplets, lysosomes in the tissue as well as abundant disintegrated myelin figures and fibrous strands. Furthermore, marked histological necrosis was found mainly at the tip of the Ommaya tube. These morphological findings corresponded to the high signal intensity areas on the gadolinium-enhanced T1-weighted MRI. These facts may indicate that the antineoplastic agents administered directly to a **tumor** per se cause morphological alternations. Moreover, these facts may suggest a therapeutic effect in the residual **tumor** cells which would be facilitated by formation of **coagulation necrosis** and collagenous tissue.

L22 ANSWER 29 OF 112 MEDLINE DUPLICATE 9
ACCESSION NUMBER: 1998071244 MEDLINE
DOCUMENT NUMBER: 98071244 PubMed ID: 9407353
TITLE: Non-surgical treatment of hepatocellular **carcinoma**
.
AUTHOR: Lin D Y; Lin S M; Liaw Y F
CORPORATE SOURCE: Liver Research Unit, Chang Gung Memorial Hospital, Chang Gung College of Medicine and Technology, Taiwan.
SOURCE: JOURNAL OF GASTROENTEROLOGY AND HEPATOLOGY, **(1997 Oct)** 12 (9-10) S319-28. Ref: 97
Journal code: 8607909. ISSN: 0815-9319.
PUB. COUNTRY: Australia
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
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LANGUAGE: English
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ENTRY MONTH: 199801
ENTRY DATE: Entered STN: 19980206
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Entered Medline: 19980127

AB A decade ago, surgery was the only satisfactory treatment modality for hepatocellular **carcinoma** (HCC), but it was limited only to selected cases. For the majority of cases of HCC, systemic chemotherapy was one of the few treatment alternatives, but provided only marginal benefit. In the past 20 years, diagnostic methods have improved to an extent that small HCC less than 1 cm can be detected. Moreover, non-surgical treatment is available, of which regional therapy has been shown to prolong patients' survival, and may even replace surgical resection in some cases. Regional therapy is indicated for the treatment of HCC when there is no extrahepatic metastasis and the patient has adequate liver function reserve, thus permitting repeated therapy. Transcatheter hepatic arterial embolization (TAE) using various embolizers has been well documented to include controlled studies. However, it is not indicated for patients with thrombosed main portal veins. Its therapeutic effect is also doubtful when the **tumour** is infiltrative in nature or is hypovascular, too large or too small. Additional chemotherapeutic agents mixed into the embolizer with lipiodol and degraded starch microspheres or styrene-maleic acid-neocarzinostatin in which chemotherapeutic agents are embedded, are used with a better response, but the survival rate has not shown significant improvement. Ultrasound-guided local injection therapy is another new method of treatment of HCC. Of these techniques, **percutaneous ethanol** injection therapy (PEIT) is widely used with excellent results for small, encapsulated **tumours** in livers with less than three HCC. **Percutaneous ethanol** injection therapy can also be used in cases with portal vein thrombosis, but it is not suitable for patients having coagulopathy or ascites. Using acetic acid, OK-432, interferon or anti-**cancer** drugs in the injection therapy shows no further benefit over ethanol alone. Transcatheter echoguided thermotherapy or cryotherapy has been reported in small series of patients, as has target therapy with **immune** or radiotherapy and conformal radiotherapy. Preliminary studies show encouraging results. Systemic therapy with either single drug or multidrugs is ineffective, with a response rate of less than 20%. Immunotherapy, such as with interferon or other cytokines, is not beneficial. Hormone therapy has not

been promising, except for treatment with tamoxifen, which has been reported to show some beneficial effect. Gene therapy is still in its infancy. In summary, recent progress in non-surgical treatment of HCC has resulted in a breakthrough of regional therapy looking quite promising. Moreover, a combination of different types of regional therapies may yield better outcomes in selected individuals.

L22 ANSWER 37 OF 112 MEDLINE DUPLICATE 11

ACCESSION NUMBER: 97252406 MEDLINE
DOCUMENT NUMBER: 97252406 PubMed ID: 9097975
TITLE: **Laser**-photosensitizer assisted **immunotherapy**: a novel modality for **cancer** treatment.
AUTHOR: Chen W R; Adams R L; Carubelli R; Nordquist R E
CORPORATE SOURCE: Physics Department, Oklahoma School of Science and Mathematics, Oklahoma City 73104, USA.. wchen@ossm.edu
SOURCE: CANCER LETTERS, **(1997 May 1)** 115 (1) 25-30.
Journal code: 7600053. ISSN: 0304-3835.
PUB. COUNTRY: Ireland
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199704
ENTRY DATE: Entered STN: 19970507
Last Updated on STN: 19970507
Entered Medline: 19970429

AB Photosensitizer-enhanced **laser treatment**, where dyes are activated in situ by lasers of appropriate wavelengths, provides highly selective tissue destruction, both spatially and temporally, through photophysical reactions. Although **laser**-sensitizer **treatment** for **cancer** can achieve a controlled local **tumor** cell destruction on a large scale, total **tumor** eradication may not be accomplished because of the incomplete local **tumor** killing or the presence of **tumor** metastases, or both. The long-term control of **cancer** depends on the host **immune** surveillance and defense systems in which both cell-mediated and humoral responses are critical. In this study we report a novel minimally invasive **cancer** treatment combining the laser photophysical effects with the photobiological effects. Irradiation of a rat mammary **tumor** by an 805 nm diode laser, after an intratumor administration of a specific photosensitizer, indocyanine green in a glycated chitosan gel, caused immediate photothermal destruction of **neoplastic** cells. Concomitantly this treatment stimulated the immunological defense system against residual and metastatic **tumor** cells. Increases in survival rate and in the eradication of **tumor** burden, both primary and metastatic, were observed after this treatment. Furthermore, the resistance of successfully treated rats to **tumor** rechallenge demonstrated a long-lasting systemic effect of the treatment. These findings indicate that our treatment has triggered a specific humoral **immune** response in the **tumor**-bearing rats.

L22 ANSWER 40 OF 112 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1996:432948 CAPLUS
DOCUMENT NUMBER: 125:80755
TITLE: Potentiation of photodynamic therapy-elicited antitumor response by localized treatment with granulocyte-**macrophage** colony-stimulating factor
AUTHOR(S): Krosl, Gorazd; Korbelik, Mladen; Krosl, Jana; Dougherty, Graeme J.
CORPORATE SOURCE: Cancer Imaging, British Columbia Cancer Agency, Vancouver, BC, V5Z 1L3, Can.
SOURCE: Cancer Research (**1996**), 56(14), 3281-3286
CODEN: CNREA8; ISSN: 0008-5472
PUBLISHER: American Association for Cancer Research
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Murine squamous cell **carcinoma** (SCCVII) cells were genetically engineered to produce murine granulocyte-**macrophage** colony-stimulating factor (GM-CSF). GM-CSF immunotherapy, based on the peritumoral injection of lethally irradiated GM-CSF-producing SCCVII cells, was examd. as adjuvant to photodynamic therapy (PDT) treatment of this **tumor**. The GM-CSF immunotherapy administered three times in 48-h intervals, starting 2 days before the light treatment, substantially improved the curative effect of Photofrin-mediated PDT. A comparable effect of GM-CSF immunotherapy was obsd. in the combination with benzoporphyrin deriv.-mediated PDT. The **tumor**-localized GM-CSF immunotherapy alone had no obvious effect on the growth of parental SCCVII **tumors**. This treatment did not significantly alter the differential peripheral WBC count and appeared not to affect **tumor** leukocyte infiltration. However, GM-CSF treatment did increase the cytotoxic activity of **tumor**-assocd. **macrophages** against SCCVII **tumor** cells. It appears, therefore, that **tumor**-localized **immune** stimulation by GM-CSF amplifies a PDT-induced antitumor **immune** reaction, which has a potentiating effect on **tumor** control.

L22 ANSWER 15 OF 112 MEDLINE DUPLICATE 4
ACCESSION NUMBER: 2000000221 MEDLINE
DOCUMENT NUMBER: 20000221 PubMed ID: 10532421
TITLE: Intracranial **malignant** lymphomas: clinicopathological study of 26 autopsy cases.
AUTHOR: Onda K; Wakabayashi K; Tanaka R; Takahashi H
CORPORATE SOURCE: Department of Neurosurgery, Brain Research Institute, Niigata University, Japan.
SOURCE: BRAIN TUMOR PATHOLOGY, **(1999)** 16 (1) 29-35. Journal code: 9716507. ISSN: 1433-7398.
PUB. COUNTRY: Japan
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199911
ENTRY DATE: Entered STN: 20000111
Last Updated on STN: 20000111
Entered Medline: 19991124

AB We examined 26 autopsy-proven cases of intracranial **malignant** lymphoma (IML) in immunocompetent patients to determine the extent of **neoplastic** involvement of the central nervous system (CNS) and to evaluate the effects of radiation on the **tumor** and brain tissue. All **tumors** were identified as diffuse non-Hodgkin's lymphomas of **B-cell** origin. In six patients who had not received radiotherapy, the clinical course of the disease was short and extensive infiltration of the **tumor** was seen. The remaining 20 patients were treated with radiotherapy and had a longer survival time. Leptomeningeal involvement was common, but extensive subarachnoid proliferation of the **tumor** was seen in only two cases. The posterior, but not anterior, lobe of the pituitary was involved in 5 of 22 cases, and choroid plexus involvement was seen in 4 of 21. Direct invasion of the **tumor** into the spinal cord, which tended to occur in patients with posterior fossa masses, was observed in 5 of 21 cases. Following irradiation, **coagulation necrosis** was frequently found in the invading zone as well as in the **tumor** mass, and degeneration of the white matter was also seen. We suggest that IML can extensively infiltrate into the CNS, including the posterior lobe of the pituitary and spinal cord, and that radiation injury to the brain appears to occur relatively easily in this disease.

L22 ANSWER 26 OF 112 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1998:383596 CAPLUS
DOCUMENT NUMBER: 129:158528
TITLE: Anti-**tumor immune** responses induced by photodynamic immunotherapy in rats
AUTHOR(S): Chen, Wei R.; Robinson, Karen E.; Adams, Robert L.; Singhal, Anil K.; Nordquist, Robert E.
CORPORATE SOURCE: Oklahoma School of Science and Mathematics, Oklahoma City, OK, 73104, USA
SOURCE: Proceedings of SPIE-The International Society for Optical Engineering (**1998**), 3254(Laser-Tissue Interaction IX), 27-34
CODEN: PSISDG; ISSN: 0277-786X
PUBLISHER: SPIE-The International Society for Optical Engineering
DOCUMENT TYPE: Journal
LANGUAGE: English
AB A new **laser immunotherapy** was used to treat metastatic mammary rat **tumors**. This new modality consists of three components: a near-IR diode laser, a photosensitizer, and an immunoadjuvant. The sensitizer-adjuvant soln. was injected directly to the **tumor**, followed by a non-invasive laser application. The new method resulted in total eradication of the treated primary **tumors** and eradication of untreated metastases at remote sites. Obsd. was the long-term survival of treated **tumor**-bearing rats: up to 120 days after **tumor** inoculation, a 300% increase in survival length compared with untreated control **tumor**-bearing rats. In addn., the successfully treated rats were refractory to **tumor** rechallenge with 10 times of the original **tumor** dose. Fluorescein and peroxidase immunochem. assays were also performed using sera from cured rats as the primary antibody. Strong antibody binding to both live and preserved **tumor** cells was obsd. Western blot anal., using the cured rat serum as primary antibody also showed distinctive protein binding, suggesting the induction of **tumor**-specific humoral **immune** response. These results indicated that an **immune** response was induced by the treatment of laser, photosensitizer and immunoadjuvant.

L22 ANSWER 22 OF 112 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1998:470354 CAPLUS
DOCUMENT NUMBER: 129:241870
TITLE: Copper steam **laser** irradiation modulates the **therapeutic** effect of 5-fluorouracil and activity of antioxidant enzymes in **tumor** -bearing mice
AUTHOR(S): Cherdyntseva, N.; Kuznetsova, A.; Kondakova, I.; Yevtushenko, V.
CORPORATE SOURCE: Oncology Institute of Tomsk Research Centre, Academy of Medical Sciences, Tomsk, Russia
SOURCE: Proceedings of SPIE-The International Society for Optical Engineering (**1998**), 3403(Atomic and Molecular Pulsed Lasers II), 290-295
CODEN: PSISDG; ISSN: 0277-786X
PUBLISHER: SPIE-The International Society for Optical Engineering
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Low-energy laser irradn. (LELI) is known to modulate some parameters of **immune** system and free radical and antioxidant reactions in organism. LELI have been shown to enhance the efficacy of chemo- and radiotherapy in exptl. models of **tumor** growth. The aim of our work was to study the ability of copper steam laser irradn. to modulate the antitumor effect of 5-fluorouracil (5-FU) and antioxidant enzyme activities in blood and **tumor** tissue of mice with transplanted Lewis lung **carcinoma**. The data obtained show that the copper steam laser irradn. is able to increase the efficacy of treatment with 5-FU. Laser irradn. increase the SOD activity in plasma and in contrary, decreases it in **tumor** tissue. Laser irradn. effect on the activity of antioxidant enzymes may be considered as one of the mechanisms mediating its ability to increase the efficacy of cytostatic therapy.

L22 ANSWER 32 OF 112 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

ACCESSION NUMBER: 1997:120088 BIOSIS

DOCUMENT NUMBER: PREV199799426591

TITLE: Treatment of hepatocellular **carcinoma**.

AUTHOR(S): Bruix, Jordi

CORPORATE SOURCE: Liver Unit, Hosp. Clin., Univ. Barcelona, Villarroel 170, 08036 Barcelona Spain

SOURCE: Hepatology, (1997) Vol. 25, No. 2, pp. 259-262. ISSN: 0270-9139.

DOCUMENT TYPE: General Review

LANGUAGE: English

L22 ANSWER 14 OF 112 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1999:307336 CAPLUS
DOCUMENT NUMBER: 131:113146
TITLE: Potential applications of photodynamic therapy
AUTHOR(S): Okunaka, Tetsuya; Kato, Harubumi
CORPORATE SOURCE: Department of Surgery, Tokyo Medical University, Tokyo, 160-0023, Japan
SOURCE: Reviews in Contemporary Pharmacotherapy (**1999**), 10(1), 59-68
CODEN: RCPHFW; ISSN: 0954-8602
PUBLISHER: Marius Press
DOCUMENT TYPE: Journal; General Review
LANGUAGE: English

AB A review with over 100 refs. At the present time, photodynamic therapy (PDT) is under active investigation for a range of therapeutic applications, in both oncol. and nononcol. areas of medicine. In oncol., a no. of studies have indicated that PDT has potential in the **laser treatment** of **malignant tumors**. In addn., PDT may be used preoperatively to increase operability, and reduce the extent of required resection, in the surgical treatment of lung **cancer**. The results of preclin. investigations have suggested that PDT may be useful in bone marrow purging to eliminate **malignant** cells prior to marrow transplantation, and clin. trials are now underway to examine this further. A particularly successful use of PDT is likely to be for the treatment of Barrett's oesophagus where **malignancy** may involve long sections of oesophagus, with multifocal and unpredictable distribution. A no. of nononcol. applications for PDT have been proposed. For example, initial studies suggest that it may reduce nonchoroidal neovascularization in ocular vascular disease. PDT has also been investigated for use in treating atherosclerotic cardiovascular lesions or to prevent restenosis following balloon angioplasty, again with encouraging results. Under certain conditions, PDT may modulate immunol. processes, as a result of the destruction of immunol. active cells; it has been proposed that this property could be utilized to relieve rheumatic symptoms by down-regulating the cellular **immune** response in rheumatoid arthritis. Selective destruction of pathol. synovium, while leaving articular surfaces undamaged, may also be achieved by appropriate use of PDT. PDT may have some applications in virol.; its uses against papilloma virus and against HIV and blood-borne viruses are still under investigation. The accessibility of skin to light offers an opportunity for the use of PDT in the treatment of dermatol. conditions; early studies suggest that it can be helpful in psoriasis, while its uses in acne, alopecia areata, portwine stains and hair removal are also being investigated. The next few years will certainly see an expansion of the indications for PDT.

REFERENCE COUNT: 86 THERE ARE 86 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L27 ANSWER 96 OF 99 CANCERLIT
ACCESSION NUMBER: 86619297 CANCERLIT
DOCUMENT NUMBER: 86619297
TITLE: EXPERIMENTAL ENU INDUCED BRAIN **TUMORS** WITH HPD AND DYE LASER LIGHT.
AUTHOR: Pezzotta S; Spanu G; Cubeddu R; Andreoni A; Giordana M T
CORPORATE SOURCE: Dipartimento di Chirurgia-Sezione di Clinica Neurochirurgia, Universita di Pavia, Italy.
SOURCE: Non-serial, **(1984)** Porphyrins in Tumor Phototherapy. Andreoni A, Cubeddu R, eds. New York, Plenum Press, p. 227-33, 1984. .
DOCUMENT TYPE: (MEETING PAPER)
LANGUAGE: English
FILE SEGMENT: Institute for Cell and Developmental Biology
ENTRY MONTH: 198602
ENTRY DATE: Entered STN: 19941107
Last Updated on STN: 19941107

AB Sixty Fisher-344 rats, including some in whom brain **tumors** had been induced by treatment of their mothers with 20 mg/kg iv ethylnitrosourea (ENU) on day 16 of gestation, were divided into three groups: one control group (Group 1) underwent **laser treatment** only, a second control group (Group 2) underwent combined treatment with hematoporphyrin derivative (HpD: 5 mg/kg, iv) and an argon ion laser using Rhodamine B (200 mW for each of two optical fibers placed on the dura, 10 min), and an ENU-treated group (Group 3) also underwent the combined treatment. For Groups 2 and 3, the **laser treatments** were begun 48 hr after HpD injection; each group received 2 **laser treatments** 3 days apart. In studies of the uptake of HpD, the HpD content of normal brain tissue was considered to be equal to zero, after allowing for traces in capillaries and blood vessels that could not be excluded from tissue homogenate; in contrast, HpD values in three different oncotypes of brain **tumors** induced with the ENU were at least 5 times higher than that in the controls: isomorphic oliogodendroglioma incorporated 0.50 gamma/g of tissue; polymorphic oliogodendroglioma, 0.72 gamma/g; and Gasser neurinoma, 0.81 gamma/g. The Group 1 rats exhibited a necrotic cyst surrounded by a moderate gliomesodermic reaction, with the lesion situated in the cortex and subcortical white matter close to the point of laser application. In the Group 2 and Group 3 rats, the gliomesodermic reaction was intense, consisting of abundant **macrophages**, vessel proliferation with endothelial hyperplasia, and hypertrophic reactive astrocytes; mitotic activity was prominent, both in the reactive elements and in the immediately surrounding glial cells. Because of the small sizes of the **tumors** at 90 days, when all animals were sacrificed, effects on the **tumors** could not be observed. (19 Refs)

L27 ANSWER 97 OF 99 CANCERLIT
ACCESSION NUMBER: 86619294 CANCERLIT
DOCUMENT NUMBER: 86619294
TITLE: HEMATOPORPHYRIN DERIVATIVE PHOTOTHERAPY IN EXPERIMENTAL ONCOLOGY.
AUTHOR: Canti G; Ricci L; Franco P; Nicolin A; Andreoni A; Cubeddu R
CORPORATE SOURCE: Dept. of Pharmacology, Sch. of Medicine, Milan, Italy.
SOURCE: Non-serial, **(1984)** Porphyrins in Tumor Phototherapy. Andreoni A, Cubeddu R, eds. New York, Plenum Press, p. 203-12, 1984. .
DOCUMENT TYPE: (MEETING PAPER)
LANGUAGE: English
FILE SEGMENT: Institute for Cell and Developmental Biology
ENTRY MONTH: 198602
ENTRY DATE: Entered STN: 19941107
Last Updated on STN: 19941107

AB Several experiments elucidating the effects of hematoporphyrin derivative (Hpd) in cultured cells and mice are described. The Hpd (0.5-50 ug/ml) caused a dose-dependent inhibition of 3H-thymidine uptake by L1210 murine leukemia cells. A similar response was exhibited by C57 spleen cells, with greater inhibition in phytohemagglutinin-stimulated cells. Hpd-treated C57 **lymphocytes**, previously sensitized to L1210 cells, had no cytotoxic activity on the relevant target cells, whereas Hpd-treated target cells showed no change in susceptibility to cell-mediated lysis. Gross or non-specific chemical modifications of cell surface molecules did not appear to be responsible for the effector cells' loss of recognition capacity. Treatment of L1210 cells with the Hpd (1, 5, and 20 ug/ml) caused an almost complete inhibition of concanavalin A agglutinability; the inhibition was dose-dependent. Light-mediated cytocidal activity was observed in the Hpd-treated L1210 cells; at a concentration of Hpd of 2.5 ug/l cell viability was 90% without He-Ne **laser treatment**, but 0% with it. Syngeneic mice challenged with 10(5) viable L1210 cells and then treated with 5 gamma/ml Hpd and laser light survived indefinitely, in spite of the fact that 10(2) cells inoculated into mice that were not subsequently treated with Hpd-Raser were lethal; Hpd treatment without laser light did not improve the survival. When CD2F1 mice bearing Moloney sarcoma virus (MS-2) sarcoma were treated with Hpd at 25 mg/kg + laser, their mean survival time was increased compared to that of animals receiving no treatment or Hpd only. Similarly, the mean survival time of BDF1 mice bearing metastatic B16 melanoma was almost doubled by treatment with Hpd x 3 + laser x 3, whereas results obtained with no treatment, Hpd-only, and laser-only were virtually the same. In the experiments with the metastatic B16 **tumor**, the laser light was delivered directly to the **tumors** by a fiber optic device embedded in the **tumor** mass. (10 Refs)

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1. Proceedings of SPIE-The international society for optical engineering:
   1999, Vol. 3601, (Laser-Tissue Interaction X), pp. 75-81
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12.
L22 ANSWER 104 OF 112 CANCERLIT

8401896.

ACCESSION NUMBER: 86619294 CANCERLIT
DOCUMENT NUMBER: 86619294
TITLE: HEMATOPORPHYRIN DERIVATIVE PHOTOTHERAPY IN EXPERIMENTAL ONCOLOGY.
AUTHOR: Canti G; Ricci L; Franco P; Nicolin A; Andreoni A; Cubeddu R
CORPORATE SOURCE: Dept. of Pharmacology, Sch. of Medicine, Milan, Italy.
SOURCE: Non-serial, (1984) Porphyrins in Tumor Phototherapy. Andreoni A, Cubeddu R, eds. New York, Plenum Press, p. 203-12, 1984. .
DOCUMENT TYPE: (MEETING PAPER)
LANGUAGE: English
FILE SEGMENT: Institute for Cell and Developmental Biology
ENTRY MONTH: 198602
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HEMATOPORPHYRIN DERIVATIVE PHOTOTHERAPY IN EXPERIMENTAL ONCOLOGY

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INTRODUCTION

Photoradiation therapy is currently under investigation as a new form of treatment for solid malignant tumors in human. The method involves photosensitization of drugs and fluorescent dyes, such as Hematoporphyrin[1]. This dye has been shown to accumulate with a certain degree of specificity in tissues with a high mitotic index[2]. Moreover, neoplastic tissue might be identified by the fluorescence emitted from cancerous lesions that have incorporated the dye in larger amounts than normal tissues[3]. The photodynamic action has implicated singlet oxygen (a short-lived excited molecular state of ground-state triplet oxygen) as the effective cytotoxic agent[4].

This property has assumed in recent years therapeutic significance due to its applicability to the treatment of experimental[5] and human tumors[6.7.8.]. Although promising applications in the therapy of superficial metastatic foci of human tumors and bronchial neoplasia[9] have been obtained by a combined treatment with Hpd and light exposure, a number of basic characteristics of Hpd, namely the optimal schedule of treatment, possible interactions with classical antineoplastic agents, toxic effects, biological alterations of normal and neoplastic cells have not yet been studied in detail.

In our laboratory we decided to study the Hpd properties in two different ways:

1. Hpd without light exposure. 2. Hpd and light exposure.
We studied the Hpd effect on immune system and its cytocidal activity with laser light.

RESULTS AND DISCUSSION

In our laboratory a long lasting, although reversible inhibition of DNA synthesis in Hpd treated leukemic cells, maintained in the dark, has been observed. (Table 1).

In these experiments, leukemic cells from L1210 murine leukemia were treated with Hpd at the indicated concentrations for 45' in the dark. ^{3}H-thymidine was added 18h before the culture sacrifice. The Hpd treatment shows an uptake inhibition of thymidine dose-dependent. These doses (also twenty times more) were not cytotoxic for the leukemic cells. The recovery of DNA synthesis is in 72h. Hpd inhibition of DNA synthesis was also observed, in a different manner, in normal and PHA stimulated lymphocytes (Table 2).

Table 1. Hpd-inhibition of ^{3}H-thymidine uptake by L1210 cells pretreated with Hod in vitro.

Hpd µg/ml	% uptake inhibition
-	0
0.5	7
1	51
2.5	68
5	80
12.5	83
25	92
50	92

L1210 cells, 10^{7}/ml were treated with Hpd at the indicated concentrations. ^{3}H-thymidine, 0.8µCi/20µl, was added 18h before the culture sacrifice. Hpd was prepared as described[7].

Table 2. Hpd-inhibition of ^{3}H-thymidine uptake by unstimulated and PHA stimulated lymphocytes.

Hpd µg/ml	% uptake inhibition PHA
-	0
0.5	48
1	49
2.5	41
5	59
12.5	77
25	97

	0
	51
	77
	79
	94
	97
	100

10×10^{6} C57 spleen cells were incubated for 1h at 37°C with Hpd diluted in HBSS at the indicated concentrations and washed in excess HBSS. 5×10^{5} cells suspended in 100 µl conditioned medium were incubated for 48h, with or without PHA, in microculture wells and [^{3}H]-thymidine was added 18h before culture sacrifice.

An almost complete inhibition of thymidine incorporation was obtained in both lymphocyte populations with the highest Hpd dose (25µg/ml). When Hpd concentration was decreased to 12.5, 5 and 2.5 µg/ml, the inhibition of thymidine incorporation is lower in resting lymphocytes than in PHA stimulated cells and this could be confirmation of the Hpd preferential incorporation by large and fast metabolizing cells such as PHA stimulated lymphocytes and neoplastic cells; this preferential incorporation has been exploited to selectively remove upon light exposure blast lymphocytes from resting lymphocytes[10].

Since immunity is important in the control of tumor growth and spreading, we decided to examine the effect of Hpd-treatment on lymphoid cells, evaluated by a number of *in vitro* assays commonly used to study humoral and cell mediated immunity. One important function of T lymphocytes is the cellular cytotoxicity that can be measured *in vitro* with the cell mediated cytotoxic assay. Constituents of the cell-mediated cytotoxic assay, namely target cells and effector cells, were pretreated with Hpd and the results of the lytic reaction are reported in Table 3.

Table 3. Effects on the CML of an Hpd treatment of effector cells or target cells.

Target cells	Effector cells	CPM ± SE	% Cytotoxicity E/T 100 : 1
L1210	* I	1027 ± 101	40.2
"	I_{H20}	589 ± 49	0.4
"	I_{H5}	652 ± 53	6.1
"	I_{H1}	890 ± 68	27.7
** $L1210_{H20}$	I	1039 ± 98	41.3
$L1210_{H5}$	"	1033 ± 96	40.8
$L1210_{H1}$	"	1049 ± 96	42.2

* C57 lymphocytes immune against L1210 cells treated with Hpd at the indicated concentrations.
** L1210 cells treated with Hpd (μg/ml).

Hpd treated C57 lymphocytes, previously sensitized to L1210 cells did not have any cytotoxic activity on the relevant target cells. Highly effective cytotoxic cells were completely inhibited as a consequence of Hpd treatment. In contrast, target cell susceptibility to cell-mediated lysis was not affected by Hpd treatment. The unchanged susceptibility to cell-mediated lysis by treated cells appears to exclude indiscriminate Hpd damage of cell surface structures. Cell mediated lysis of target cells proceeds through two fundamental steps: the recognition phase and the lethal hit phase. Table 4 shows that effector recognition was inhibited while the relevant characteristic of target cells to be recognized was not modified. Inhibition was dose-dependent and the viability of neither the effector cells nor the target cells was damaged.

In other experiments of absorbing capacity and lysis susceptibility we did not observe any Hpd alteration of target molecules like surface receptors. Gross and non specific chemical modifications of cell surface molecules seem not be responsible for the effector cell loss of recognition capacity.

Table 4. Effect of an Hpd treatment on immune lymphocyte binding to target cells.

Target cells	Immune Lymphocytes	No. of positive cells ± SE	% binding
* L1210	** I	242 ± 29	48
"	I_{H20}	90 ± 18	18
"	I_{H5}	136 ± 9	27
"	I_{H1}	222 ± 12	44
$L1210_{H20}$	I	255 ± 28	51
$L1210_{H5}$	"	240 ± 20	48
$L1210_{H1}$	"	250 ± 23	50

* see footnote Table 3 ** see footnote Table 3

^ Immune cells and target cells in a ratio of 40:1 incubated 1h at 4°C were counted under a light microscope by two independent observers. Fivehundred small lymphocytes were counted and cells with 3 or more tumor cells bound were considered positive cells.

Another set of experiments reported in Table 5 were directed at seeing whether Hpd might influence the lateral mobility of molecular structures on cell surface with the Con A agglutination of cancer cells test.

Hpd treatment of the cells caused an almost complete inhibition of lectin agglutination. Inhibition was dose-dependent. Studies are in progress to elucidate the nature of membrane modifications and, possibly, the specific structure damage. The inhibition of some immune cell activities reported here appeared to be dependent on Hpd alterations of the movement of cell surface structures rather than an alteration of the chemical integrity of cell surface molecules, since the intimate mechanism of many cellular interactions is still obscure, detection of specific Hpd alteration of membrane properties might help to elucidate some characteristics of immune cell interations and, more generally, to study some properties of eukaryotic cells.

Table 5. Reduced Con A agglutinability of Hpd treated L1210 cells.

Tumor cells	Con A µg/ml	Agglutination index
L1210	50	++++
$L1210_{H2O}$	"	+
$L1210_{H5}$	"	$+\pm$
$L1210_{H1}$	"	$+\pm$
L1210	100	++++
$L1210_{H2O}$	"	$\pm$
$L1210_{H5}$	"	+
$L1210_{H1}$	"	+

2×10^6 Hpd treated L1210 cells were incubated with Con A (50-100 µg/ml) 30' at 37°C and then 10' at 4°C. The agglutination index was evaluated under the light microscope by two independent observers with arbitrary scores from 0 to ++++.

Regarding the results of Hpd activity on tumor cells after light activation with laser, Hpd treated leukemic cells, undamaged in the dark, have been completely lysed upon exposure to the red light of an He-Neon laser (Table 6).

Light mediated cytocidal activity was dependent on Hpd concentration. A low Hpd dose such as 2.5 µg/ml devoid of any cytotoxic activity in the absence of light, exhibited the maximum cytocidal effect.

Table 6. Cytocidal effect of Hpd and laser on L1210 tumor cells.

Hpd μg/ml	Cell viability - Laser	Cell viability + Laser	Specific ^{51}Cr release - Laser	Specific ^{51}Cr release + Laser
-	87	91	0	0
0.5	85	87	3	1
1	91	38	1	44
2.5	90	0	4	86
5	85	0	3	91
12.5	73	0	11	93
25	68	0	14	93

L1210 cells treated with Hpd at the indicated concentrations, after extensive washing were exposed for 45' to an He-Neon laser light or maintained in the dark. Cell viability was checked by the Trypan blue dye exclusion test. In a parallel experiment, $Na_2{}^{51}CrO_4$, 200 μCi/ml (specific activity 200mCi/mM, Amersham England) was added to samples under treatment with Hpd, washed and treated with laser as described. Specific ^{51}Cr release in supernatant by Hpd-laser-treated samples was calculated as follows:

$$\frac{\text{CPM in experimental samples - CPM in control samples}}{\text{CPM in detergent treated samples - CPM in control samples}} \times 100$$

The potent cytocidal activity of light activated Hpd was further confirmed by injection of Hpd-laser treated L1210 cells in syngeneic animals and data are reported in Table 7.

The time of death of syngeneic animals challenged with Hpd-laser-treated L1210 cells was registered. The large majority of mice challenged with viable 10^5 L1210 cells treated with 5γ/ml and light survived indefinitely, in spite of the fact that an L1210 inoculum as low as 10^2 cells is lethal. In contrast the survival of L1210 leukemic mice was not improved by the Hpd treatment alone.

Table 7. Survival of mice challenged with L1210 cells pre-treated with Hpd and laser.

HPD γ/ml	Laser − MST	Laser − D/T	Laser + MST	Laser + D/T
-	10	8/8	10	8/8
50	12	8/8	-	0/8
25	11	8/8	-	1/8
12.5	11	8/8	-	1/8
5	9	8/8	-	2/8
2.5	10	8/8	12	7/8
1	10	8/8	12	8/8

L1210 cells, untreated or treated with Hpd were maintained in the dark or exposed to laser light as indicated for Table 6. $CD2F_1$ mice were challenged with 10^5 cells ip and the time of death registered. MST, median survival time (days); D/T, dead animals/treated animals.

The Hpd-laser cytocidal activity *in vivo* was studied in mice bearing MS-2 sarcoma derived from murine Moloney Sarcoma Virus (MSV-M) (Table 8).

Table 8. Antitumor activity of Hpd + Laser

Treatment	Exp.1 Hpd 5mg/kg ip MST	Exp.1 Hpd 5mg/kg ip D/T	Exp.2 Hpd 25mg/kg ip MST	Exp.2 Hpd 25mg/kg ip D/T
-	43	6/6	46	6/6
HPD	45	6/6	49	6/6
HPD X3 * LASER X3	45	6/6	89	6/6
HPD + LASER	45	6/6	69.5	6/6
LASER	52	6/6	ND	

$CD2F_1$ mice challenged id with 10^6 sarcoma MS-2.
* = 50mW/cm^2 x45'.

Treatment of mice with Hpd and exposure of the tumor mass to laser light prolonged the median survival time in respect to the control animals untreated or treated with Hpd or with laser (dye-laser) P=50mW /cm^2 x 45'only. The problem regarding relapse around the scar area is probably due to some microscopic neoplastic cells that had already spread into normal tissues. As presently applied however, the major limitation of this method is the limited effective penetration of the light through tissues, estimated to be approximately 2 cm. This limitation can be eliminated by delivering the light through a quartz fiber optic, imbedded directly into the tumor mass. Indeed results using this technique are the same as other external techniques.

After these encouraging results we decided to carry out some experiments with another solid tumor, the B16 melanoma, that quickly metastatizes to the lung. Infact, in clinical oncology, the metastases are the most important problem, therefore we tried to apply this new methodology on one experimental metastatic tumor. For these experiments the laser light was delivered through a quartz fiber optic imbedded directly into the tumor mass. (Table 9).

We observe from this table that the phototherapy is quite active on the metastatic tumor.

In conclusion these preliminary results are encouraging in view of the clinical applications. Studies are in progress to compare

Table 9. Antitumor activity of Hpd + laser on a tumor with established metastases.

x Treatment	MST	D/T
	23	6/6
xx Hpd	25	6/6
xx Laser	24	6/6
Hpd x 3 + Laser x 3	46	5/6

BDF_1 challenged id with 10^6 B16 melanoma. x Treatment was started when tumor had lung metastases. xx 200 mW (fiber output) x 10'.

the photoradiation methodology with the conventional surgical excision, to explore new and eventually more active cancer therapies and furthermore to combine different modalities of treatment.

These studies, infact, might improve the efficacy of the photoradiation therapy and, more important, could be interesting for better clinical approaches.

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EFFECT OF PHOTODYNAMIC THERAPY ON ANTI-TUMOR IMMUNE DEFENSES: COMPARISON OF THE PHOTOSENSITIZERS HEMATOPORPHYRIN DERIVATIVE AND CHLORO-ALUMINUM SULFONATED PHTHALOCYANINE

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Abstract—The effects of the two photosensitizers chloroaluminum sulfonated phthalocyanine (ClAlSPc) and hematoporphyrin derivative (HpD) on the functional activities of macrophages and natural killer (NK) cells, two immunocyte populations implicated in the control of tumor development and spread, have been investigated. Murine peritoneal macrophages treated *in vivo* with ClAlSPc or HpD at 10 mg/kg body weight showed no impairment of Fc-mediated phagocytic capacity and only minor disturbances of *in vitro* tumoricidal/tumoristatic function. The NK cell activity of splenocytes obtained from photosensitizer-treated mice, assayed 24 or 48 h after i.v. injection of ClAlSPc or HpD at 10 mg/kg was unaffected compared to controls. However significant inhibition of NK activity was observed when splenocytes obtained from mice with or without subcutaneous Colo 26 tumors, treated with ClAlSPc plus laser therapy (675 nm) were used as effector cells. The results show that impairment of some anti-tumor activity can be observed in phthalocyanine treated or phthalocyanine + laser-treated animals but this relatively minor impairment may augur well for the use of systemic phthalocyanine administration in photodynamic therapy.

INTRODUCTION

Photodynamic therapy (PDT)† of cancer is based on the systemic administration of photosensitive dyes followed by exposure of the site of tumor growth to light of a particular wavelength (Doiron and Keller, 1986). The parenteral delivery of any anti-neoplastic agent theoretically could produce a wide range of systemic effects, including the type of immune suppression frequently seen following administration of chemotherapeutic drugs (Mihich, 1975).

Surprisingly there have been few studies on the effect of photosensitizer dyes, with and without light delivery, on host anti-tumor defenses. The commonly used photosensitizing dye hematoporphyrin derivative (HpD) has been shown to diminish the blastogenic response of lymphocytes to the mitogen PHA (Canti *et al.*, 1981) and to inhibit the cytotoxic efficacy of T lymphocytes and natural killer cells in *in vitro* assays (Franco *et al.*, 1983). The combination of HpD plus light exposure abrogated the ability of peripheral blood mononuclear cells to act as stimulator cells in a mixed lymphocyte reaction and prolonged graft survival of transplanted tail skin (Gruner *et al.*, 1985). Additionally, HpD decreased dinitrofluorobenzene-induced hypersensitivity; an effect which has been attributed to the existence of a putative suppressor cell population (Elmets and Bowen, 1986). Most recently Gomer *et al.* (1986) have shown that a complete, though transitory, suppression of natural killer (NK) cell activity can be achieved by treatment of mice with the dihematoporphyrin ether-enriched form of HpD in combination with laser light of 630 nm. Thus though the literature on the effect of HpD and HpD-mediated PDT is sparse there are suggestions that the effects of such treatments may be deleterious for specific host immune defense mechanisms.

We (Chan *et al.*, 1987; 1988) and others (Ben-Hur and Rosenthal, 1985; Brasseur *et al.*, 1985; Tralau *et al.*, 1987), have proposed that the porphyrin-like phthalocyanine dyes may prove to be useful photosensitizers in PDT. However, as far as we can ascertain, no work has been done on the interaction of phthalocyanine dyes with natural anti-tumor immune mechanisms. In the present studies the effects of ClAlSPc on functional activities of macrophages and NK cells, two immunocyte populations implicated in the control of tumor development and spread (Eccles, 1978; Normann, 1985; Hanna, 1982; 1985), have been evaluated and compared with the effects of HpD in the same assays.

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†*Abbreviations*: CCD, charge-coupled device; ClAlSPc, chloro-aluminum sulphonated phthalocyanine; DEM, Dulbecco's modification of Eagle's minimal medium, supplemented with 10% (v/v) fetal calf serum and glutamine; HpD, hematoporphyrin derivative; IdUrd, 5-[^{125}I]Iodo-2'-deoxyrudine; i.p., intraperitoneal; i.v., intravenous; NK, natural killer; PDT, photodynamic therapy; PEC, peritoneal exudate cells; PBS, phosphate buffered saline; SRBC, sheep red blood cells.

MATERIALS AND METHODS

Animals. C57BL/Icrf and BALB/c/Icrf mice were used in this study. Adult female mice 8–16 weeks old and weighing approximately 25 g were obtained from the Specific-Pathogen-Free breeding unit of the Imperial Cancer Research Fund laboratories. Standard food and water was provided *ad libitum*.

Photosensitizers. HpD was prepared by R. Svensen, The Royal Institution, London, according to the method of Lipson *et al.* (1961). ClAlSPc was obtained from Ciba-Geigy Dyestuffs and Chemicals (Basel, Switzerland). Stock solutions of 5 mg/mℓ were stored at 4°C in the dark until use when they were diluted to working strength and filtered through a 0.22 μm filter.

Cell lines and culture conditions. Four murine tumor lines were used in the studies. UV-2237 is an UV-induced fibrosarcoma syngeneic to C3H mice (Kripke *et al.*, 1978), Colo-26 is a colorectal carcinoma of BALB/c mice (Tsuro *et al.*, 1983) and B16F10 is a high lung-colonizing line of the B16 melanoma syngeneic to C57BL mice (Fidler, 1973). These three adherent lines were cultured on plastic in Dulbecco's modification of Eagle's minimal medium supplemented with 10% heat-inactivated fetal calf serum and L-glutamine (DEM). Yac-1, a Moloney virus-induced T-cell lymphoma of A/Sn mice (Cikes *et al.*, 1973) was grown as a suspension culture in RPMI–1640 medium supplemented as above. Cultures were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2.

Mice received i.p. injections of 2–4 mℓ thioglycollate broth (Becton Dickinson, U.K.) or 0.25 mℓ (of 1mg/mℓ *Corynebacterium parvum* in normal saline (Wellcome Research Laboratories, Kent, U.K.) 5 days prior to cell collection. Exudate cells were harvested by peritoneal lavage with 6–8 mℓ of ice-cold EDTA [0.01% wt/vol in phosphate buffered saline (PBS, pH 7.2)], pelleted and resuspended to final concentration (viability > 95% as assessed by trypan blue exclusion) in RPMI–1640 plus 20% fetal calf serum. Use was made of the adherence capacity of macrophages to enrich this exudate suspension for macrophage populations (Edelson and Cohn, 1976). Briefly cells in suspension were allowed to adhere to tissue culture plastic substrates for 2 h at 37°C before non-adherent cells were rinsed away. By morphological criteria, after May-Gruenwald/Giemsa or non-specific esterase (Van Furth *et al.*, 1979) staining, such populations were > 95% macrophages.

Fc-mediated phagocytosis of opsonized sheep erythrocytes. Sodium ^{51}Cr-chromate labeled sheep red blood cells were used to determine Fc-receptor-mediated phagocytosis. The assay was performed in LP3 tubes (Luckham Ltd., Sussex, U.K.) following the method described by Talmadge *et al.* (1981).

Macrophage-mediated cytotoxicity assays. Macrophage-mediated effects on tumor cells were evaluated using two assay systems (1) a radioisotope based technique and (2) a spectrophotometry method. The radioisotope technique was based on the [^{125}I] IdUrd release procedure as described elsewhere (Fidler *et al.*, 1981).The spectrophotometry method was essentially that detailed by Bennett *et al.* (1986). Briefly, non-radiolabeled target cells were suspended at 5×10^4/mℓ in DEM and aliquots were added to the wells of 96-well Microtiter II dishes containing pre-plated effector macrophages to give effector:target ratios of 20:1 and 10:1. Following 72 h incubation at 37°C, assays were terminated by washing away non-adherent cells with three washes of PBS, cells were fixed in methanol for 10 min and air dried. One hundred microliters of crystal violet solution (0.1% wt/vol) was added to each well for 10 min, plates were then washed repeatedly with tap water until washings were colorless and stored dry until read. Stain was eluted by the addition of 100 μℓ 33% acetic acid (vol/vol solution) and absorbance at 620 nm was measured on a Titertek Multiskan (Flow Laboratories, U.K.). Quadruplicate samples were read for each effector:target cell ratio and standard deviations were ±5% of the mean. All assays were performed under darkened conditions (see below).

Natural killer (NK) cell activity. The 4 h chromium release assay was used to determine the NK cell activity of murine splenocyte effector cells against ^{51}Cr-labeled YAC-1 lymphoma cells (Bennett *et al.*, 1986). All assays were performed in darkened conditions (see below).

Administration of photosensitizer dyes. In specific experiments (as detailed in the Results section) mice received i.p. or i.v. injections of ClAlSPc or HpD at a dose rate of 10 mg/kg (based upon 25 g mouse) and the various effector cell populations were harvested 24 h or 48 h later and used in the appropriate assays. In these instances great care was taken to minimize exposure to incident light and assays were set-up in a darkened tissue culture hood. Plates were loosely wrapped in aluminum foil during culture to exclude light.

Evaluation of NK cell activity after ClAlSPc or HpD-mediated photodynamic therapy. BALB/c mice (2 per group), bearing Colo-26 tumors growing in the s.c. site of the left inguinal region, were given i.p. injections of ClAlSPc or HpD (10 mg/mℓ) or control injections of PBS 24 h or 48 h prior to laser irradiation.

Animals were anesthetized with i.p. barbiturate ('Sagatal', May and Baker, Dagenham, U.K.; 175 μℓ at 6 mg/mℓ) before being immobilized, ventral side up, with surgical tape. The left inguinal region was swabbed with ethanol and a 19 gauge needle used to pierce the skin and the tumor capsule. A single 0.2 mm diameter quartz fiber, covered with plastic to within 1 mm of the tip, was inserted into the hole so that the fiber tip was located at the center of the tumor nodule. Laser light (675 nm for ClAlSPc-treated mice, 630 nm for HpD treated mice) was delivered through the fiber from an argon ion pumped dye laser (Aurora-Cooper, Lasersonics). The dye used was DCM (4-Dicyanomethylene-2-methyl-6-(p-dimethylaminostyrl)-4-H pyran) in ethylene glycol and propylene carbonate. Photoirradiation was for 33 min at a power of 50 or 100 mW giving 100 or 200 J total energy. In one experiment, non-tumor bearing mice were treated in an identical fashion with the insertion of the quartz fiber tip approximately 2–3 mm below the skin surface (Expt. 3, Table 2). To control for the effects of laser therapy animals received a dye and were anesthetized as described above but photoirradiation was not conducted (data not shown).

Twenty four hours after irradiation mice were killed by CO_2 inhalation and the spleens from 2 mice per group were pooled. Natural killer cell activity of pooled splenocytes was determined using the assay described above.

RESULTS

Phagocytic capacity of peritoneal exudate cells derived from photosensitizer-treated mice

The results from one of two very similar experiments are presented in Table 1. Mice received either 0.5 mℓ PBS or 0.5 mℓ of ClAlSPc or HpD at 10 mg/kg in PBS i.p. 24 h prior to exudate cell collection. At this dose by flow-cytometric analysis, charge-coupled device (CCD) camera analysis or spectrophotometric measurement of cell lysates we have shown high uptake of the dyes by peritoneal exudate cells following this route of administration (data not shown). Nonetheless the presence of either dye in the macrophages had no discernible effect on their ability to phagocytose opsonized sheep red blood cells (SRBC) (Table 1).

Table 1. Phagocytic capacity of peritoneal exudate cells from photosensitizer-treated mice

Mouse	PBS*	ClAlSPc	HpD
1	4921 ± 476	5965 ± 842	7157 ± 272
2	6894 ± 796	6844 ± 777	6420 ± 588
3	6510 ± 488	6922 ± 1108	6236 ± 652
	6108 ± 1046†	6577 ± 531	6604 ± 487

^{51}Cr-labeled SRBC were sensitized with anti-SRBC. Data presented are radioactivity (in cpm) of quadruplicate samples ± standard deviation.
*Material injected i.p. (0.5 mℓ volumes) 24 h prior to peritoneal exudate cell collection.
†No significant difference between mean pooled values from three individual mice, Student's *t*-test.

Anti-tumor efficacy of peritoneal macrophages obtained from photosensitizer-treated mice

Representative data for a spectrophotometry assay using B16-F10 and UV-2237 target cells are presented in Fig. 1. The amount of stain taken up by fixed cells increases with the number of tumor cells present and results are expressed by plotting optical density *vs* Log_{10} (PEC dilution)$^{-1}$. The effector:target cell ratio which caused a 50% reduction in the O.D. relative to control wells (i.e. tumor cells growing in the absence of macrophages) was determined and used to calculate the relative tumoricidal/tumoristatic activity of the various macrophage populations. The data given in Fig. 1 are taken from a single experiment of five similar experiments. Individual macrophage populations showed similar reactivity against both UV-2237 and B16-F10 cells. The anti-tumor activity of macrophages from individual mice within the same treatment group appeared quite variable when photosensitizer treatment was used in contrast to the similar behavior of macrophages from control animals. Thus in the data presented in Fig. 1 one out of three animals treated with ClAlSPc and two out of three animals treated with HpD exhibited impaired macrophage reactivity against both B16-F10 and UV-2237 target cells. However over the five separate individual experiments no consistent pattern of significant impaired efficacy was established; a finding that was supported by the data derived from [^{125}I] IdUrd cytotoxicity assays (data not shown).

Effect of photosensitizer treatment on NK cell activity

The NK cell activity of C57BL mouse splenocytes, determined by the 4 h ^{51}Cr-release assay using

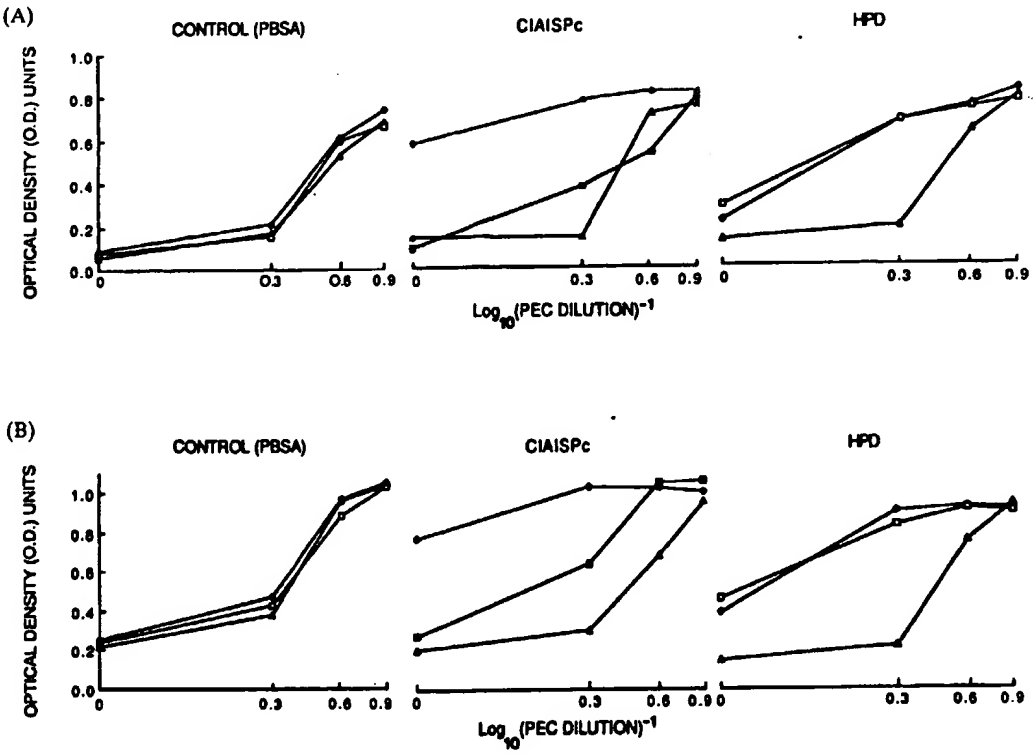

Figure 1. Macrophage mediated inhibition of tumor cell growth at various effector:target cell ratios. (A) UV-2237 fibrosarcoma cells as targets; (B) B16-F10 melanoma cells as targets. Mice were treated with PBS, ClAlSPc or HpD as detailed in the text. Points on graph are mean of triplicate samples (± standard deviations of <5%) and represent values obtained from three individual animals per group. Assays terminated after 72 h incubation.

YAC-1 lymphoma cells, was not reduced 24 or 48 h after the i.v. administration of ClAlSPc or HpD at a dose of 10 mg/kg (Fig. 2).

Effect of photodynamic therapy on NK cell activity

Three experiments were performed to assess the effect of PDT on NK cell activity in ClAlSPc-treated mice and the results are presented in Table 2. All animals, both control and dye-treated, had tumors growing at the subcutaneous site in Experiments 1 and 2 and all animals in Experiment 3 were non-tumor bearers (Table 2).

Relative to the NK cell activity of control splenocytes tested on the same day the combination of ClAlSPc administration and laser light irradiation reduced significantly the cytotoxic capacity of murine splenocytes in the 4 h ^{51}Cr-release assay (Table 2). In two similar experiments conducted with HpD as the photosensitizing agent we failed to demonstrate any reduction in NK cell potency (data not shown).

DISCUSSION

In the present studies we have examined the effects of two agents which may prove useful in the photodynamic therapy of cancer, the photosensitizers HpD and ClAlSPc, on the functional capacities of two immunocyte populations, macrophages and NK cells, which are thought to play a pivotal role in regulating tumor growth and spread (Eccles, 1978; Hanna, 1985). In addition to the need to evaluate macrophage function because of its likely role in tumor development we thought it important to examine this cell type because its phagocytic capacities make it a likely site of photosensitizer localization *in vivo* (Moan, 1986). Indeed recently we have shown, by disaggregation techniques and flow cytometric analysis, that macrophages infiltrating the Colo-26 tumor take up large quantities of ClAlSPc (Chan *et al.*, 1988). Moreover under *in vitro* conditions macrophages derived from the peritoneum accumulate 2–3 times more HpD or ClAlSPc than do syngeneic tumor cells (data not shown).

The ability of macrophages to take up large amounts of photosensitizer has been confirmed by our examination of peritoneal exudate cells by fluorospectrophotometry, subsequent to the i.p. delivery of ClAlSPc or HpD (data not shown).

In spite of this capacity for photosensitizer accumulation functional activities of macrophage populations derived from dye-treated animals

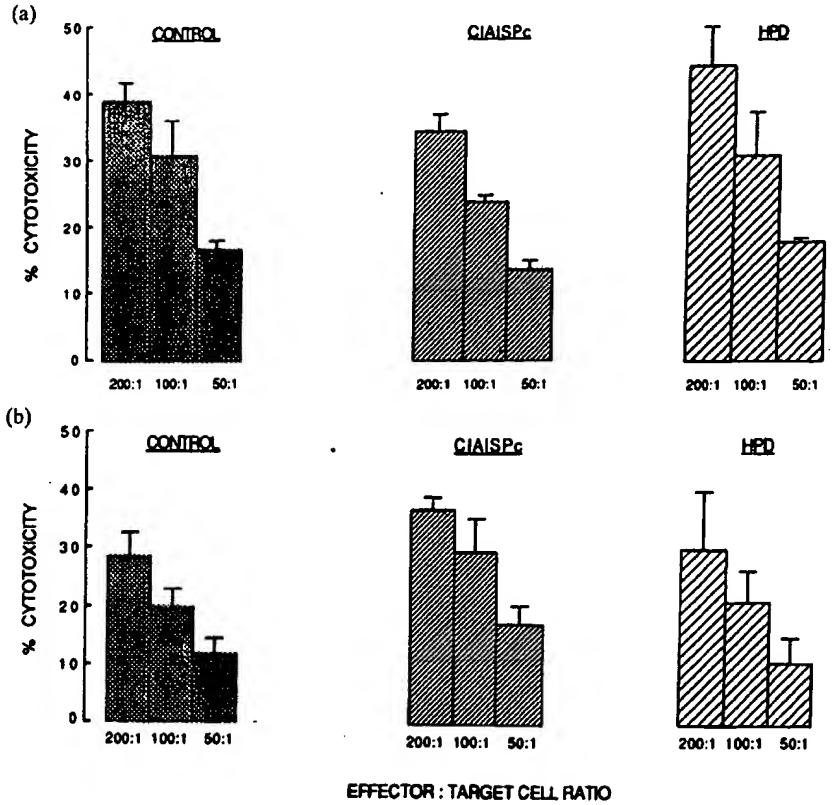

Figure 2. NK cell mediated cytotoxicity of splenocytes obtained from mice 24 (a) or 48 h (b) after PBS, ClAlSPc or HpD administration. Target cells were ^{51}Cr-labeled Yac lymphoma. Values given are means from three individual mice per group at the various effector:target cell ratios; vertical bars represent one standard deviation.

Table 2. Effect of photodynamic therapy on NK cell activity

		% Cytotoxicity at effector:target ratio:		
		100:1	50:	25:1
Exp. 1†	Control	26.9 ± 3.1	22.3 ± 1.5	15.4 ± 0.7
	ClAlSPc	19.9 ± 1.7*	14.2 ± 0.2*	4.2 ± 0.1*
Exp. 2†	Control	16.6 ± 0.5	11.3 ± 0.4	5.5 ± 0.6
	ClAlSPc	10.8 ± 0.8*	4.6 ± 0.2*	4.5 ± 0.4
Exp. 3‡	Control	30.0 ± 1.5	24.1 ± 1.2	18.4 ± 0.9
	ClAlSPc	15.3 ± 1.0*	10.5 ± 0.3*	7.0 ± 0.4*

Three independent experiments performed on different days.
Percentage cytotoxicity values are means ± SD of quadruplicate samples; effector cells from pooled splenocytes from two mice per treatment group.
*$P \leq 0.05$ by Student's *t*-test comparing control and ClAlSPc treatment groups within single experiment.
†All animals had s.c. growing tumor.
‡Neither group of animals had s.c. growing tumors.

remain relatively unimpaired. A series of experiments failed to show any adverse effect of either HpD or ClAlSPc on macrophage phagocytic capacity as determined by the ability to engulf opsonized SRBCs (Table 1). Since Fc-mediated phagocytosis represents an important line of defense against infection (Roitt, 1984) and many cancer patients have infectious complications as sequelae of the underlying malignancy and its treatment (Pizzo and Young, 1985) this result suggests that photosensitizer treatment may be unlikely to add to such complications.

The observations on the anti-tumor cytotoxic and cytostatic effects of macrophage populations after dye uptake are rather more equivocal. Only data from the spectrophotometric assay, whose results give an inseparable indication of both cytotoxicity and cytostasis (Flick and Gifford, 1984; Bennett *et al.*, 1986) are provided, but similar findings were obtained with the [^{125}I]-IdUrd-release assay. Overall no consistent significant suppression of macrophage activity was observed but, as in the experiment presented in Fig. 1, it often was noted that within treatment groups macrophages from individual mice could vary markedly in terms of their anti-tumor potency; a variation that commonly was not detected between mice in the control group (Fig. 1). We have shown that under room light conditions HpD is toxic to cells which have incorporated this substance and this could explain why such variability was observed in the HpD-treated group since in spite of our precautions it may have proved impossible to exclude all activating light (Chan *et al.*, 1986). However, such an explanation is unlikely to account for heterogeneity in the anti-tumor response of macrophages from ClAlSPc-treated mice since room light does not activate this photosensitizer (Chan *et al.*, 1986). While over the ten individual experiments performed there seems to have been no regular marked reduction in anti-tumor cell functional capacity it seems reasonable to conclude that both HpD and ClAlSPc are capable of impairing the tumor-restricting activity of macrophage populations to a limited extent.

The second major population of immunocytes investigated in the present studies were splenic NK cells. In murine models at least, the NK cell system has been implicated strongly in the elimination of circulating tumor cells and in the restriction of tumor development (Hanna, 1985). Photosensitizer treatment alone did not affect the NK cell activity of splenocytes recovered from treated mice (Fig. 2). However when ClAlSPc administration was coupled with laser light administration there was significant suppression of NK cell activity with, at the 50:1 effector:target cell ratio, values from treated animals ranging from 36.4% to 59.3% of control values (Table 2). The number of animals used in these studies was small and absolute levels of cytotoxicity varied from day to day but the concordance of three separate repetitions makes it probable that this effect is real. The combination of dye and light might result in the systemic release of a factor(s) capable of suppressing NK cell activity. Alternatively, if NK cells recirculate through the body like lymphocytes (Gowans, 1959), suppression may be mediated by contact with suppressive factors at a distant site or by exposure to laser light during passage through the treatment area. These possibilities currently are under investigation in our laboratory. In direct contrast to other studies (Gomer *et al.*, 1986) we failed to show any suppression of NK cell activity by HpD-dependent PDT. However, the differences in experimental design such as the different mouse strains used and the exact nature of the photosensitizer used could well account for these discrepancies; ClAlSPc for example absorbs red-light more strongly than HpD.

Systemic administration of any compound could have marked effects on the immune system; particularly a substance which is retained preferentially in cells of the mononuclear phagocyte lineage, as are

the two photosensitizers used in the present studies. It is therefore promising that ClAlSPc, a phthalocyanine which has produced dramatic responses in transplantable tumors at the doses used in the studies reported here (Chan *et al.*, 1987; 1988) shows relatively minor effects against some anti-tumor effector mechanisms and these results might augur well for clinical application of ClAlSPc in PDT of human tumors.

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CLINICAL LASER PHOTODYNAMIC THERAPY IN THE TREATMENT OF BLADDER CARCINOMA

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Abstract—The treatment of bladder carcinoma using dihematoporphyrin ether (DHE) and laser photodynamic therapy (PDT) is described herein. Patients selected for this study have cytology- and biopsy-proven transitional cell carcinoma, no histologic evidence of muscle invasion, and negative excretory urograms.

Sixteen patients have been treated, with follow-up from 6 to 36 months. Eleven have had a complete response, and 3 a partial response in that they required re-treatment for recurrence. Two of these patients have not recurred at this time. One of the patients who recurred had tumor extension into the prostatic urethra and has been successfully re-treated (disease-free at 6 months). There was one treatment failure and 1 patient lost to follow-up.

Photosensitivity for up to 4 weeks is a known side-effect, but unexpected morbidity included a transient but significant increase in urinary frequency, urgency, and occasionally hematuria which spontaneously resolved within 3–4 weeks. Careful placement of the fiberoptic tip in the centre of the bladder, bladder distension during treatment with saline rather than water, the instillation of the minimum volume required to "smooth out" the mucosa for complete bladder photoradiation, and delivered energy of 25 J cm^{-1} or less may have prevented the more severe complications (i.e. bladder shrinkage) reported by Dougherty and Nseyo (personal communication).

We also feel that there is some early evidence that a heightened immune response (similar to intravesical BCG) may potentially play some role in explaining the efficacy of PDT in long disease-free intervals, although this is just a histologic observation at present.

It appears the PDT offers another practical treatment modality for non-invasive transitional cell carcinoma in patients refractory to standard surgical and chemotherapeutic regimens, and has been addressed by numerous other investigators such as Benson (1985) and Hisazumi (1983). We are presently recommending to our patients in these categories to undergo a course of PDT prior to relinquishing to cystectomy.

INTRODUCTION

The treatment of bulky, superficial transitional cell carcinoma, frequent recurrences of new tumors, and the presence of carcinoma *in situ* have always presented problems in the rational management of non-invasive bladder neoplasia. Repeat transurethral resection of visible lesions is the most common treatment. Intravesical instillation of a variety of chemotherapeutic agents (i.e. Adriamycin, Thiotepa, etc.) is yet another method of management, although its overall success in preventing long-term recurrent disease is less than 50%. Promise has been shown in stimulating the body's own immune system via intravesical BCG (Bacillus Calmette–Guerin), and this may yet prove to be an effective modality. At present, however, the morbidity with these treatment regimens over time for the individual patient is very significant. In spite of this, a number of these patients with high-grade disease will ultimately need to undergo bladder extirpation.

We are presently involved with a therapeutic modality using i.v. administration of dihematoporphyrin ether (DHE) followed by Rhodamine B dye pumped argon laser light at 632 nm. DHE is a purified active agent found in hematoporphyrin derivative (HpD). Use of this photosensitizing agent and laser photodynamic therapy (PDT) has given us promising clinical results for those patients with carcinoma *in situ* and/or aggressive superficial disease who seem to be destined for surgical extirpation via radical cystectomy.

Photodynamic therapy is a photo-initiated oxidation involving energy transfer from the DHE molecule to form singlet oxygen as a reactive short-lived intermediate. It appears that singlet oxygen initially involves changes in the cell membranes, resulting in cellular destruction, and this can be detected via ultramicroscopic techniques as early as 10 min after sensitizing light exposure. Additionally, there also appears to be some direct effect on the surrounding vascular bed which may enhance tumor destruction.

MATERIALS AND METHODS

The clinical use of DHE is still experimental and follows specific FDA-approved guidelines in a multicenter protocol set administered by Photomedica, Inc. (1986). Patients are selected for the present set of protocols if they meet the following criteria:

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(1) biopsy-proven, non-invasive transitional cell carcinoma of the bladder;
(2) negative excretory urography to rule out simultaneous upper tract involvement;
(3) patients with carcinoma *in situ*; or
(4) patients with repeat tumor recurrence within 18 months; or
(5) patients in whom all the visible tumor present cannot be resected due to physical constraints; and
(6) patients who agree to take part in the protocol fully, including initial randomization to either intravesical Thiotepa administration or PDT.

We have found that careful explanation of the protocol to the patient and family members, along with viewing of a prepared videotape presentation, will make the patient much more comfortable with the entire informed consent process. This is often neglected, but we feel its importance cannot be overemphasized. Patient fears, complaints, and anxieties are markedly decreased.

After informed consent has been obtained, the drug is injected intravenously at a dose of 2 mg kg^{-1}. Due to secondary uptake of the DHE in the skin, significant photosensitivity occurs, and patients must remain in a light-subdued room prior to treatment. Although there is marked variability in uptake time which may vary based on tissue type, we have allowed 72 h after injection for maximal concentration differential by the tumor. The patient is then taken to the operating suite. Although there is very little discomfort from the treatment *per se*, it is difficult for both the patient and the treating physician to maintain good comfort and keep the treatment tip centered without anesthesia. We have found that light general or short-acting spinal anesthesia to be very satisfactory.

Light delivery is through a quartz fiber optic bundle passed through the side part of a standard cystoscope with a double port bridge. A special fiberoptic treatment tip resembling a miniature bulb allows us to flood the entire bladder with light at an isophotometrically delivered dose of 25 J cm^{-1}. This tip is used routinely; we have used the microlens tip for focal disease on one occasion and have found it to be of limited value. A modified test-tube holder attached to a steel rod clamps the cystoscope firmly in place for rigid positioning, and a ureteral catheter is used to measure the distance from the vesical neck to the posterior wall–dome area. The bulb tip should be positioned in the center of the bladder to allow equal light distribution to all areas of the bladder. The ureteral catheter is then withdrawn back into the scope. Every 10–15 min saline is gradually drained or added as needed till the ureteral catheter again measures the same pre-treatment distance to the bladder wall. Light exposure time to yield this dosage is calculated based on the laser output and bladder volume and averages about 38 min.

Following the patient's hospital discharge the following day after dusk, protection from direct sunlight must be maintained for *ca.* 4 weeks. Artificial lighting presents no problem, but daytime excursions should be avoided if at all possible. Very few of our patients have experienced any difficulty, but review of our data shows we tend to treat primarily in the winter months, when daylight hours are shorter and the risk of incidental exposure are somewhat minimized. We have not found that over the counter sunscreen preparations are of value, since none of them block out all wavelengths which can cause tissue damage, and have the disadvantage of lulling the patient into a false sense of security.

Follow-up for patients is quite standard. Repeat endocystoscopy, random bladder biopsies, and urine cytologies are performed every 3 months for the first year, every 6 months for the second year, and yearly thereafter. Although protocol insists that we obtain urine cytologies, we have noticed little correlation between the apparent findings at the first follow-up visit and cold cup biopsy material. This appears to be due to the cytologist's difficulty in differentiating degenerating or inflammatory cells and true neoplasia soon after PDT. Indeed, a number of patients with "positive" cytologies at 3 months later became negative. Thus, we feel that cytology, at least initially, is a poor method of follow-up.

RESULTS

At this writing we have treated 16 patients. Follow-up has been from 6 to 36 months. Twelve patients had diffuse carcinoma *in situ* (Tis, Grade III intraepithelial neoplasia). Four patients had Ta, Grade II–III papillary transitional cell carcinoma and had recurred multiple times. Two of these patients were visible tumor free at the time of treatment and 2 had some residual tumor. Of the four that recurred, all have been re-treated. Two of these had 12 and 15 months respectively between recurrences and remain disease free. Another patient remained tumor free in the bladder, but developed disease in the prostatic urethra. He was treated with a cylindrical tip fiber with a delivered dose of 100 J cm^{-1}. He remains disease free at 6 months. One patient was considered a failure in that he recurred a third time, but the area of involvement was confined to one small area of the bladder and he has remained disease free after Nd:YAG laser ablation of the area (4 months). One patient was lost to follow-up immediately post-PDT.

DISCUSSION

Ongoing discussion with other investigators shows wide variance in treatment parameters (light dosage, amount of drug injected, etc.) One disturbing complication reported has been that of bladder shrinkage with significant irritative symptoms. We have not had this complication in our patients, other than a transient (<4 weeks) but quite intense increase in frequency, urgency, and mild hematuria which spontaneously resolved. This may be due to several factors. First, we limit bladder distension prior to treatment to the minimum volume required to "smooth out" the bladder lining (usually about 150 cm^3) using saline, rather than water. Second, total power delivered to the bladder mucosa does not exceed 25 J cm^{-1}. Finally, we have not treated any patient who has previously had external beam bladder radiation.

Although the patient complaints of urgency and frequency in the immediate post-PDT treatment period are significant, a variety of medications generally not used for bladder symptoms give good relief. Ibuprofen, a non-steroidal anti-inflammatory agent, and narcotic analgesics (i.e. meperidine) afford some relief, while traditional anti-cholinergics (propantheline, oxybutynin) appear to offer little or no relief. Severity of symptoms seem to have some correlation with recurrence; those with minimal or no bladder symptoms seem to recur soonest. Patients are now told that the symptoms are actually a "good" sign and this reassurance does much to

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Because of the relationship between these symptoms and recurrence rate, we began to look at other associated factors. It seemed unusual that patients obtained some relief from anti-inflammatory agents, when urologists rarely prescribe these for routine bladder irritability in other conditions. Besides the evidence that those patients with the least subjective complaints tended to recur earlier, a number of patients have gone as long as 3 years without a recurrence. Indeed, if the presumed mechanism of PDT efficacy is primarily cytotoxicity, one would expect recurrence within 6 months (approximate time for full-thickness cell turnover). This can possibly be explained on the basis of full thickness mucosal slough, and this most likely plays a major role in long disease-free intervals. Additionally, routine surveillence biopsies on disease-free patients invariably come back with a diagnosis of "chronic cystitis", although there is no evidence of infection or obvious inflammation. Microscopic examination shows normal mucosa with an unusually large number of chronic inflammatory cells present—lymphocytes, plasma cells, and histiocytes. Immunoperoxidase staining of biopsy specimens seems to show an increase in T cell lymphocytes and plasma cells, although cross-comparison and control studies have not been completed at the time of this writing.

Although much clinical immunologic work needs to be done, the possibility of some role played by a heightened local immune response (similar to intravesical BCG) may help explain the efficacy of PDT.

Quite obviously, it will be some time before we fully understand the basic mechanisms of PDT activity in neoplasia. What is clear is that DHE PDT shows great promise as a therapeutic tool for carcinoma *in situ* and aggressive, superficial disease. We are presently recommending those people that fulfill the protocol criteria to have a course of DHE PDT prior to relinquishing to cystectomy.

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